REMARKS

Amendments to the Specification

The "Related Applications" section has been updated.

The specification has been further amended to complete the renumbering of the figures in view of the deletion of Figure 8. No new matter has been added.

Drawings

Corrected Figures 1-37E are being submitted concurrently herewith. No new matter has been added.

Attached hereto is a marked-up version of the changes made to the application by the current amendment. The attached page is captioned "Version with Markings to show changes made." Also attached hereto is a copy of all the pending claims. This attached page is captioned "Pending Claims."

Priority

The Examiner states that the priority application, U.S.S.N. 07/431,565 (filed November 3, 1989) does not disclose the full length sequence of SEQ ID NO:6. Applicants respectfully traverse this rejection.

The instant case claims priority to, among other applications, International Application Serial No. PCT/US90/06548 (filed November 2, 1990). Support for the full length sequence of SEQ ID NO: 6 can be found throughout PCT/US90/06548, *e.g.*, at least in Figure 3 (a copy of PCT/US90/06548 is enclosed as Appendix A). Accordingly, the instant case is at least entitled to claim priority to the filing date of PCT/US90/06548 (November 2, 1990).

Double Patenting

Claims 101-104 are rejected under the judicially created doctrine of obviousness-type double patenting as "being unpatentable over claims 1-19 of U.S. Patent No. 6,048,962." Applicants respectfully traverse this rejection. However, to expedite prosecution, Applicants submit herewith a Terminal Disclaimer which

disclaims any portion of the patent issuing from the above-referenced application that extends beyond the patent term of U.S. Patent No. 6,048,962. Accordingly, the rejection is now moot.

Claims 95, 96, 101, 103, and 104 are rejected under the judicially created doctrine of obviousness-type double patenting as "being unpatentable over claims 1-26 of U.S. Patent No. 6,019,972." Applicants respectfully traverse this rejection. However, to expedite prosecution, Applicants submit herewith a Terminal Disclaimer which disclaims any portion of the patent issuing from the above-referenced application that extends beyond the patent term of U.S. Patent No. 6,019,972. Accordingly, the rejection is now moot.

Rejection of Claims 95, 96, 101, 102, and 104 Under 35 U.S.C.§102(a)

Claims 95, 96, 101, 102, and 104 are rejected under 35 U.S.C. §102(a) "as being anticipated by Morgenstern *et al.* [June 17, 1991]" Applicants respectfully disagree.

The instant case claims priority to, among other applications, International Application Serial No. PCT/US90/06548 (filed November 2, 1990). Support for claims 95, 96, 101, 102, and 104 can be found throughout PCT/US90/06548. Specifically, support for the full length sequence of SEQ ID NO: 6 can be found in at least Figure 3 of PCT/US90/06548 (a copy of PCT/US90/06548 is enclosed as Appendix A). Accordingly, the instant case is at least entitled to claim priority to the filing date of PCT/US90/06548 (November 2, 1990). Accordingly, Morgenstern *et al.* is not a prior art reference and claims 95, 96, 101, 102, and 104 are novel.

Rejection of Claims 95, 96, and 101-103 Under 35 U.S.C.§102(b)

Claims 95, 96, and 101-103 are rejected under 35 U.S.C. §102(b) "as being anticipated by Littler *et al.*" In particular, the Examiner states that a portion of the MCP protein described in Littler *et al.* (residues 354-360) share 100% identity with SEQ ID NO:6 (residues 103-109). The Examiner further states that the recombinantly produced peptides produced antibodies reactive with HHV-6. From this, the Examiner concludes that "the peptides [described in Littler *et al.*] meet the structural limitations of the claims and are administered to animals producing an immunogen specific response, the

reference teachings inherently anticipate a therapeutic composition comprising the peptide." Applicants respectfully traverse this rejection.

The peptides used for producing antibodies in Littler *et al.* did not include a peptide consisting of residues 354-360 of the MCP protein (see Figure 1 of Littler *et al.*). Accordingly, Littler *et al.* fail to teach or suggest residues 354-360 as encompassing an epitope. Since the pending claims require that the polypeptide include an epitope, Littler *et al.* fail to teach or suggest the claimed invention.

Further, as known in the art at the time of the present invention, epitopes involve at least seven (7) amino acid residues (see, for example, Livingstone *et al.* (1987) Ann. Rev. Immunol. 5:477-501 at page 482; enclosed as Appendix B). Accordingly, residues 354-360 of the MCP protein fail to meet he basic definition of an epitope-containing peptide.

Based on at least the following, claims 95, 96, and 101-103 are novel in view of the cited reference.

Rejection of Claims 95, 96, and 101-104 Under 35 U.S.C.§103(a)

Claims 95, 96, and 101-104 are rejected under 35 U.S.C. §103(a) as "not patentably distinct from claims 1-19 of commonly assigned U.S. Patent No. 6,048,962 and from claims 1-26 of commonly assigned 6,019,972."

Applicants respectfully traverse this rejection. However, to expedite examination, Applicants submit herewith a Declaration pursuant to 37 C.F.R. §1.132 signed by the current Assignee (Heska Corporation) stating that the above-referenced application and U.S. Patent Nos. 6,048,962 and 6,019,972 are assigned to Heska Corp. Heska Corp. acquired its right in the present application and in U.S. Patent Nos. 6,048,962 and 6,019,972 by way of assignment from Immulogic Pharmaceutical Corporation. In the Declaration, Heska Corp. further states that, in accordance with 37 CFR §1.78(c), the invention claimed in the present application and the inventions described and claimed in U.S. Patent Nos. 6,048,962 and 6,019,972 were, at that time the invention claimed in the present application was made, owned or subject to an obligation of assignment to the same entity, Immulogic Pharmaceutical Corporation. Accordingly, this rejection is now moot.

Rejection of Claims 95, 96, and 101-104 Under 35 U.S.C.§103(a)

Claims 95, 96, and 101-104 are rejected under 35 U.S.C. §103(a) as "being unpatentable over 6,048,962 and 6,019,972." Applicants respectfully traverse this rejection. However, to expedite examination, Applicants submit herewith a Declaration pursuant to 37 CFR §1.132 signed by the current Assignee (Heska Corporation) stating that the above-referenced application and U.S. Patent Nos. 6,048,962 and 6,019,972 are assigned to Heska Corp. Heska Corp. acquired its right in the present application and in U.S. Patent Nos. 6,048,962 and 6,019,972 by way of assignment from Immulogic Pharmaceutical Corporation. In the Declaration, Heska Corp. further states that, in accordance with 37 CFR §1.78(c), the invention claimed in the present application and the inventions described and claimed in U.S. Patent Nos. 6,048,962 and 6,019,972 were, at that time the invention claimed in the present application was made, owned or subject to an obligation of assignment to the same entity, Immulogic Pharmaceutical Corporation. Accordingly, this rejection is now moot.

Rejection of Claim 103 Under 35 U.S.C.§103(a)

Claim 103 is rejected under 35 U.S.C. §103(a) as "being unpatentable over Morgenstern *et al.* in view of Sibson *et al.*" As described above, the substance of which is reiterated here, Morgenstern *et al.* is not a prior art reference. Further, Sibson *et al.* fail to cure the deficiencies of Morgenstern *et al.* Sibson *et al.* discuss cloning totally different proteins (brain adrenal tissue, placenta, and bone marrow) than those encompassed by the pending claims. Accordingly, the pending claims are patentable in view of the cited references.

Rejection of Claim 104 Under 35 U.S.C.§103(a)

Claim 104 is rejected under 35 U.S.C. §103(a) as "being unpatentable over Littler *et al.* in view of Hirschmann *et al.* (U.S. Patent No. 3,846,399)." As described above, the substance of which is reiterated here, Littler *et al.* fail to teach the subject matter encompassed by the pending claims. Further, Hirschmann *et al.* fail to cure the deficiencies of Morgenstern *et al.* Hirschmann *et al.* merely describe a generic method

for synthesizing polypeptides. Hirschmann *et al.* fail to mention anything concerning therapeutic compositions, let alone therapeutic compositions which comprise specific polypeptides as currently claimed. Accordingly, the pending claims are patentable in view of the cited references.

SUMMARY

In view of the amendments and remarks set forth above, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney would be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

Respectfully submitted,
LAHIVE & COCKFIELD, LLP

Jeanne M. DiGiorgio
Registration No. 41,710
Attorney for Applicants

28 State Street
Boston, Ma 02109
(617) 227-7400

Date: 29 January 03

Version with Markings to Show Changes Made

In the Specification:

On page 1, delete all the information under the "Related Applications" section has been deleted and replaced with the following paragraph:

-- This application is a continuation of U.S. Application No. 08/431184, filed on April 28, 1995 (now U.S. Patent No. 6,120,769; issued September 19, 2000), which is a divisional application of U.S. Application No. 08/300928, filed on September 2, 1994 (now U.S. Patent No. 6,019,972; issued February 1, 2000), which is a continuation-in-part of U.S. Application No. 07/807529, filed on Dec. 13, 1991 (now U.S. Patent No. 5,547,669; issued August 20, 1996). This application is also a continuation-in-part of U.S. Serial No. 08/006116, filed January 15, 1993 (now abandoned), which is a continuation-in-part of U. S. Serial No. 07/884718, filed May 15, 1992 (now abandoned), which is a continuation-in-part of U.S. Serial No. 07/857311, filed March 25, 1992 (now abandoned), which is a continuation-in-part of U.S. Serial No. 07/662276, filed February 28, 1991 (now abandoned), which claimed priority to International Application Serial No. PCT/US90/06548, filed November 2, 1990, and which is a continuation-in-part of U.S. Serial No. 07/431565, filed November 3, 1989 (now abandoned). The contents of the above applications are incorporated herein by reference. --

On page 5, lines 11-12 have been replaced as follows:

-- Figure ~~10~~ 9 is a graphic representation of the secondary T cell response of peripheral blood lymphocytes from patient 131 stimulated with various antigens and peptides. --

On page 7, lines 14-16 have been replaced as follows:

--Fig. 29 is the nucleic acid sequence (utilizing E. coli expression codons) and the deduced amino acid sequence comprising peptide YZX ~~(SEQ ID NOS:94 AND 95)~~ (SEQ ID NOS:102 and 103) A thrombin cleavage site is shown.--

On page 7, lines 19-20 have been replaced as follows:

-- Fig. ~~32~~ 31 is the nucleic acid sequences of primers used in the construction of peptides XZY, YXZ, and ZXY. --

On page 7, lines 32-34 have been replaced as follows:

-- Fig. ~~37~~ 36 is a graphic representation depicting responses of murine T cells immunized with peptide YZX and analyzed for response in vitro to culture with the peptide YZX as measured by IL-2 production. --

On page 63, lines 15-23 have been replaced as follows:

-- For both the secondary IL-2 and the secondary IL-4 assays (Figure ~~16~~ 15), the average number of counts from the triplicate 150 μg/ml peptide Y wells was divided by the average number of counts from the wells without peptide Y to determine a stimulation index. This was necessary because the no antigen background in the secondary cultures is more variable than in primary cultures. The peptide tolerization decreased the peptide specific production of both IL-2 and IL-4 in these secondary *in vitro* cultures. These data suggest that tolerization with peptide Y decreases the antigen specific production of both IL-2 and IL-4. The effect indicates a tolerogenic effect by administration of peptide Y on both classes of T helper cells. --

#17 3-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Malcolm L. Gefter *et al.*

Serial No.: 09/662784

Filed: September 15, 2000

For: HUMAN T CELL REACTIVE FELINE PROTEIN (TRFP) ISOLATED FROM HOUSE DUST AND USES THEREFOR

Attorney Docket No.: IMI-044DV3CN

Group Art Unit: 1647

Examiner: Turner, S.

#17

Commissioner of Patents
Washington, DC 20231

Certificate of First Class Mailing (37 CFR 1.8(a))

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on the date set forth below.

29 Jan 03
Date of Signature and of Mail Deposit

By: Jeanne M. DiGiorgio
Jeanne M. DiGiorgio
Reg. No. 41,710
Attorney for Applicants

TERMINAL DISCLAIMER

Dear Sir:

The undersigned hereby submits this terminal disclaimer on behalf of Heska Corporation, 1613 Prospect Parkway, Fort Collins, CO 80525, the assignee of all rights in the above-identified application, as evidenced by an Agreement dated December 13, 1999 between the original Assignee (ImmuLogic Pharmaceutical Corp.) and Heska Corp. Evidence that ImmuLogic Pharmaceutical Corp. is the original Assignee of the above-referenced application can be found in the Assignment document executed by each of the inventors of the above-referenced application to ImmuLogic Pharmaceutical Corp., 610 Lincoln Street, Waltham, MA 02154, recorded at the U.S. Patent Office on November 18, 1994 at Reel/Frame 7213/0170.

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02/05/2003 SDENBOB1 00000055 09662784
02 FC:1814 110.00 CH

Pursuant to 35 U.S.C. §253 and 37 C.F.R. §1.321, Heska Corp. ("Assignee") hereby disclaims the terminal portion of any patent granted on the above-identified application, which would extend beyond the expiration date of the full statutory term of U.S. Patent No. 6,048,962, entitled "Human T cell reactive feline protein (TRFP) isolated from house dust and uses therefor," or U.S. Patent No. 6,019,972, entitled "Peptides of human T cell reactive feline protein (TRFP)." Assignee also agrees that any patent so granted on the present application shall be enforceable only for and during such period that the legal title to said patents shall be the same as the legal title to U.S. Patent Nos. 6,048,962 and 6,019,972, this agreement to run with any patent granted on this application and to be binding upon the grantee, its successors or assigns.

Assignee does not disclaim any terminal part of any patent granted on the above-identified application prior to the expiration date of the full statutory term of U.S. Patent Nos. 6,048,962 or 6,019,972, in the event that U.S. Patent Nos. 6,048,962 or 6,019,972 expires for failure to pay a maintenance fee, is held unenforceable, is found invalid, is statutory disclaimed in whole or terminally disclaimed under 37 C.F.R. §1.132(a), has all claims canceled by a reexamination certificate, or is otherwise terminated prior to expiration of its statutory term as presently shortened by any terminal disclaimer, except for the separation of legal title stated above.

Respectfully submitted,

Lahive & Cockfield, LLP

Jeanne M. DiGiorgio
Attorney for Applicants
Registration No. 41,710

28 State Street
Boston, MA 02109
Tel. (617) 227-7400

Dated: **January 29, 2003**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Malcolm L. Gefter, *et al.*

Serial No.: 09/662784

Filed: September 15, 2000

For: HUMAN T CELL REACTIVE FELINE PROTEIN (TRFP) ISOLATED FROM HOUSE DUST AND USES THEREFOR

Attorney Docket No.: IMI-044DV3CN

Group Art Unit: 1647

Examiner: Turner, S.L.

#14
B.G.J
2/12/03

Commissioner for Patents
Washington, DC 20231

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231 on the date set forth below.

29 January 03
Date of Signature and of Mail Deposit

By: Jeanne M. DiGiorgio
Jeanne M. DiGiorgio
Reg. No. 41,710
Attorney for Applicants

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

I, Carol Talkington Verser, a citizen of the United States, hereby declare as follows:

(1) I am Executive Vice President of Intellectual Property and Business Development for Heska Corporation, located at 1613 Prospect Parkway, Fort Collins, CO 80525, U.S.A.

(2) Heska Corporation is the current owner of the above-referenced application and U.S. Patent No. 6,048,962 (entitled "Human T cell reactive feline protein (TRFP) isolated from house dust and uses therefor") and U.S. Patent No. 6,019,972 (entitled "Peptides of human T cell reactive feline protein (TRFP)"). Heska Corporation acquired its right in U.S.S.N. 09/662784 and U.S. Patent Nos. 6,048,962 and 6,019,972 by way of assignment from Immulogic Pharmaceutical Corporation.

(3) In accordance with 37 CFR §1.78 (c), the invention claimed in the present application and the inventions described and claimed in U.S. Patent Nos. 6,048,962 and 6,019,972 were, at that time the invention claimed in the present application was made, owned or

subject to an obligation of assignment to the same entity, Immulogic Pharmaceutical Corporation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

28 January 2003
Date

Carol Talkington Verser
Carol Talkington Verser, Ph.D.

APPENDIX B

Ann. Rev. Immunol. 1987. 5 : 477–501

THE STRUCTURE OF T-CELL EPITOPES

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INTRODUCTION

The humoral and cellular arms of the adaptive immune response, mediated by B and T lymphocytes respectively, differ fundamentally in the way in which they recognize antigen. The B-cell receptor for antigen (immunoglobulin) can bind to soluble antigen in a manner similar to that of many well-characterized receptor-ligand systems. In contrast, the T-cell antigen receptor can generally recognize antigen only in association with cell surface molecules encoded by the major histocompatibility complex (MHC). This requirement, known as MHC restriction, ensures that T-cell activation or effector function occurs only in an appropriate cellular context. Antigen-specific T-cell activation thus results from the formation of a ternary complex involving the T-cell receptor (TcR), nominal antigen, and class-I or class-II MHC molecules (1, 2).

Characterization of the antigen-specific receptors on T-helper and cytotoxic cells unfortunately did not suggest any structural basis for the MHC-restricted recognition of antigen. On the contrary, the genes encoding the α and β chains of the TcR heterodimer showed marked homology with immunoglobulin genes, and similar mechanisms of DNA rearrangement appeared to be involved in the generation of both T- and B-cell repertoires (3–5). Experiments to determine the mechanism underlying MHC restriction have therefore increasingly focused on the way in which nominal antigen might interact with MHC molecules to form a single antigenic structure recognized by the TcR.

This review is concerned with the characterization of antigenic determinants seen by T cells. Almost all the studies in this area have concentrated on the response of MHC class II (Ia)–restricted T cells to soluble

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protein antigens, for several reasons. In the first place, many of these antigens, such as lysozyme, cytochrome *c*, and myoglobin, belong to sets of homologous proteins with known primary amino acid sequence and tertiary structure. Residues that were important for T-cell stimulation could be identified by analyzing the response to several members of a protein family and comparing their sequences. Secondly, T-cell-helper/proliferative responses to many protein antigens were shown to be specific for sequential determinants. This meant that the epitopes seen by these T cells could be identified relatively easily using proteolytic fragments or synthetic peptides. In addition, the response to many of these antigens was under immune response (*Ir*) gene control. The determinant selection theory argued that Ir gene effects were determined by the ability of antigen to form an appropriate immunogenic association with Ia molecules on the antigen-presenting cell surface (6-8); many groups have therefore sought to identify T-cell epitopes on these proteins, in order to analyze the specificity of antigen-Ia interactions.

The recognition of antigens such as viruses, or minor histocompatibility antigens by class I–restricted T cells (predominantly of cytotoxic phenotype) was until recently thought to be qualitatively different from the way in which class II–restricted T cells recognized soluble protein antigens. It was assumed that these antigens were integral membrane proteins associated in some way on the cell surface with the appropriate MHC molecule. The principal reason for thinking this was that cytotoxic T cells could kill targets not known to have any antigen-processing capability. Several problems arose with this model; perhaps the most important was the almost universal failure to block cytotoxic responses with antisera specific for the nominal antigen, although antibodies specific for the class I restricting molecules could block lysis very effectively (9). Townsend and colleagues have recently shown, however, that class I–restricted cytotoxic T cells can recognize fragments of molecules that are not integral membrane proteins (10). They found that an influenza-specific, class I–restricted cytotoxic-T-cell clone was specific for the viral nucleoprotein. Most importantly, this clone could be activated by a short synthetic peptide of nucleoprotein, presented on the surface of uninfected cells expressing the appropriate MHC class-I molecules. These experiments suggest that the recognition of viral proteins by class I–restricted cytotoxic T cells may in all essentials be similar to the recognition of soluble protein antigens by class II–restricted T-helper cells (11).

In this review, we address in detail the structure of the epitopes recognized by helper T cells. The observed differences between such epitopes and those recognized by B cells are examined, and a hypothesis to explain the utility of such distinct recognition systems is presented.

T-CELL EPITOPES CAN BE DEFINED BY PRIMARY AMINO ACID SEQUENCE

The first evidence that T-cell recognition of antigen was qualitatively different from that of B cells came from experiments by Gell & Benacerraf (12), at a time when lymphocytes had not yet been divided into T- and B-cell subsets. They compared humoral and cellular responses in guinea pigs to a series of protein antigens and found that while the antibody response was specific for the form of antigen used for immunization (native versus denatured), delayed type hypersensitivity (DTH) responses (later shown to be T-cell dependent) could be elicited equally well using either native or denatured antigen. They proposed that while the antigenic structures seen by antibodies depended on the tertiary configuration of the immunizing protein, the cells effecting the DTH response might recognize epitopes sufficiently defined by primary amino acid sequence. Sela (13) had named such antigenic determinants "sequential epitopes" to distinguish them from "conformational epitopes," dependent on tertiary protein structure.

Subsequent experiments with a wide range of protein antigens supported the contention that T cells of helper/proliferative phenotype recognized denatured antigens. Thompson et al (14) showed that although there was essentially no serological cross-reaction between reduced, carboxymethylated hen egg lysozyme (RCM-HEL) and native HEL, splenic lymphocytes from guinea pigs immunized with either native HEL or RCM-HEL proliferated strongly to both forms of this antigen. Ishizaka et al (15) found that denatured ragweed antigen E could induce a specific T-helper response, even though the denaturation had destroyed the IgE-binding properties of this antigen. Similarly, Schirrmacher & Wigzell (16) showed that, while bovine serum albumin (BSA) denatured by methylation no longer bound BSA-specific antibodies, T cells from mice immunized with BSA could provide help when challenged with methylated BSA. Direct evidence that T cells could recognize sequential determinants came from experiments in which cleavage fragements of proteins such as staphylococcal nuclease, cytochrome *c*, and lysozyme were shown to stimulate T cells from animals immunized with the native protein (17–19). Finally, an important experiment by Chesnut et al (20) demonstrated that native and denatured forms of ovalbumin primed the same population of T-helper cells.

These results all suggested that protein antigens were denatured and perhaps enzymatically cleaved, prior to recognition by T cells. Studies by Rosenthal and colleagues (6, 7) had shown that the T-cell response to insulin was critically dependent upon an interaction between antigen and

macrophages of the appropriate strain. It was suggested that this macrophage-dependent step was in fact a requirement for antigen denaturation/degradation ("antigen processing") by the macrophage. This was confirmed in a series of experiments (20, 22, 23), reviewed recently by Unanue (21), where lysosomotrophic agents such as chloroquine and ammonium chloride (which raise lysosomal pH and thus inhibit proteolytic activity), or fixation of antigen-presenting cells with glutaraldehyde or paraformaldehyde, were used to block antigen processing. Shimonkevitz et al (23) showed that ovalbumin-specific T-cell hybridomas were unable to respond to native or to denatured ovalbumin presented on glutaraldehyde-fixed macrophages but could respond to proteolytic digests or cyanogen bromide fragments of ovalbumin presented by these cells—a result suggesting that cleavage of this antigen was essential for T-cell recognition. Other experiments indicated that cleavage was not invariably necessary for the effective presentation of all protein antigens. Allen & Unanue (24) analyzed the antigen processing requirements of two T-cell hybridomas, both specific for hen egg lysozyme (HEL). The first clone behaved like the ovalbumin-specific clones described above: Fixed antigen presenting cells, or cells incubated with chloroquine, could only stimulate with enzymic digests of HEL, and not with native HEL or HEL denatured by reduction and carboxymethylation (CM-HEL). The second clone, however, could be stimulated by CM-HEL (but not by native HEL), presented either by fixed cells or in the presence of chloroquine. This suggested that unfolding of the molecule was sufficient to expose the epitope seen by this particular T-cell hybridoma. Similar results were obtained by Streicher et al (25), who showed that a T-cell clone specific for sperm whale myoglobin could respond to denatured but intact myoglobin presented by cells treated with a variety of lysosomal inhibitors. Streicher et al argued that the function of antigen processing was to expose hydrophobic residues hidden inside the native protein, so that they could interact with Ia on the antigen-presenting cell surface. They suggested that proteolytic cleavage was an effective, if rather unsubtle, method of unfolding proteins to expose hydrophobic surfaces.

The objection has been raised that treatment with lysosomal inhibitors such as chloroquine, or fixation with aldehydes, may have important effects on cellular metabolism. Evidence suggests that lysosomal drugs can inhibit both the recycling of intracellular vesicles (26) and endosome fusion (27). The fact remains, however, that T cells can respond to antigen "processed" to some extent (either by denaturation or by cleavage) in circumstances where they are unable to respond to native antigen. Walden et al (28) have reported that proteins such as insulin do not necessarily need to be processed before they can be recognized by T cells. They found that native antigen incorporated into Ia-bearing lipid vesicles was able to stimulate antigen-specific T cells. They could not, however, rule out the possibility that the antigen had been denatured during incorporation into the vesicles. The clearest demonstration of a requirement for antigen processing comes from the experiments of Watts et al (29), who showed that an ovalbumin-specific hybridoma could respond to a peptide digest of ovalbumin but not to native ovalbumin when both were presented on planar membranes containing only lipid and purified Ia.

While T cells of helper/proliferative phenotype generally see processed antigen, not all T cell epitopes can be defined solely by primary amino acid sequence. Barcinski & Rosenthal (31) showed that the T cell epitope involving the α loop of the insulin A chain was destroyed if the disulfide bond holding this loop together was reduced. More recently, Naquet et al (30) have shown that insulin-specific T cell clones recognize an epitope consisting of the intact α loop of the A chain, disulfide bonded to a portion of the B chain. There was no significant stimulation of these T-cell clones by oxidized insulin A chain or B chain. Thus, the T-cell epitope(s) seen by these clones involved two separate disulfide-bonded peptides, one of which included an intrachain disulfide-bonded loop. This observation appears, however, to be the exception rather than the rule. Hen egg lysozyme, for instance, has four internal disulfide bonds, yet short peptides based on primary amino acid sequence have been able to stimulate T-cell proliferation in all cases examined so far.

STRATEGIES FOR IDENTIFYING T-CELL EPITOPES

T-cell epitopes on protein antigens have generally been identified in one of two ways. The first method works only for homologous series of proteins, such as lysozymes, insulins, myoglobins, and cytochromes, where the amino acid sequences from several members of the protein family are known. Residues important for T-cell activation can be identified by correlating the ability to stimulate a T-cell response with amino acid sequences. Alternatively, proteins can be cleaved either enzymatically or with reagents such as cyanogen bromide, and the fragments tested to see whether they stimulate a T-cell response. Epitope assignments made by either of these methods can be confirmed using synthetic peptide analogues of the sequence in question.

A number of antigenic sites were identified in this way on proteins such as insulin (31, 32), staphylococcal nuclease (17), cytochrome *c* (18, 33), myoglobin (34–36), and ovalbumin (37). Several important points could be made from these experiments. In the first place, the T-cell response was

often exquisitely specific for the immunizing antigen. In some instances, homologous proteins or peptides differing from the immunizing protein at only a single position were unable to stimulate a T-cell response (18, 31). Second, it was found that a protein could have distinct antigenic sites which stimulated different functional T-cell subsets. An analysis of the response to hen egg lysozyme in a "nonresponder" strain demonstrated that T-suppressor cells were directed against a site involving the N- and C-terminal portions of the molecule, while T-helper cells were specific for determinants in the middle and C-terminal cyanogen bromide cleavage fragments (38, 39). In addition, MHC-disparate strains were found to respond preferentially to particular antigenic regions (31, 39–43).

Over the last few years, a number of important questions about the nature of the interaction between the T-cell receptor, antigen and the restricting MHC molecule have been answered using synthetic peptides to analyze the requirements for T-cell activation. The minimum peptide length necessary for T-cell stimulation has been determined using overlapping sets of synthetic peptides, and the importance of particular residues has been examined using peptides substituted at various positions. These studies, together with analyses of the effect of peptide length on antigenic potency, have led to the suggestion that the ability of peptides to adopt appropriate secondary structure may be of crucial importance for antigenicity. In addition, comparison of known epitopes has allowed the formulation of several hypotheses about what makes a particular sequence antigenic for T cells. The repeated observation that one immunodominant region can stimulate T-cell responses that are extremely heterogeneous in fine specificity emphasizes the importance of using T-cell clones, rather than uncloned T-cell lines, for these studies. Very recently, interactions between antigenic peptides and MHC molecules have been demonstrated directly using a number of biophysical techniques. These studies are discussed in more detail in the remainder of this review.

SIZE OF T-CELL EPITOPES

T-cell epitopes on protein antigens or polypeptides seem in general to involve at least seven amino acids. The first evidence for this came from experiments with the synthetic polypeptide poly-L-lysine. Schlossman (46) analyzed the proliferative response of lymphocytes from guinea pigs immunized with poly-L-lysine and found that oligomers with seven contiguous L-lysine residues, such as [(L-lysine)$_7$,D-lysine,L-lysine (L_7DL)], were stimulatory, while oligomers of the same length and amino acid composition but with fewer than seven adjacent L-lysines (e.g. L_4DL_4), were unable to

stimulate proliferation. Further experiments in this system (47) demonstrated that T cells were responsible for the proliferative response and confirmed that peptides shorter than seven amino acids were unable to stimulate proliferation.

Similar results have since been obtained for T-cell epitopes on native proteins. The same general approach described below was used in all these experiments. Sets of overlapping peptides synthesized by serially removing one amino acid from either end were generated and tested for their ability to stimulate the T-cell clone or hybridoma in question. This strategy has been successfully applied to the fine specificity mapping of epitopes on a number of globular protein antigens (48–53). The consensus is that these T-cell epitopes contain approximately seven amino acids. It should be pointed out that since these peptides are synthesized from the C-terminus, it is very much easier to make sets of peptides where the C-terminus is constant and the N-terminus varied than it is to make peptides where the C-terminus is varied. For this reason, the N-terminal limits of these epitopes have frequently been more precisely defined than the C-termini.

Experiments in this laboratory highlight some of the complications involved in this kind of analysis. We have used sets of peptides shortened one residue at a time from both the N- and C-termini to map an epitope on sperm whale myoglobin to the seven amino acid sequence 112–118 (53). Clone 9.4 (made by A. J. Infante) responded well to peptide 110–121, progressively less well to peptides 111–121 and 112–121, and not at all to peptide 113–121. We therefore concluded that residue 112 was essential for stimulation. To test whether peptide 113–121 failed to stimulate simply because it was too short, this experiment was repeated with a longer set of peptides, where the C-terminus was kept constant at residue 124 instead of 121. The results were identical: Peptide 112–124 was stimulatory, while peptide 113–124 was not. Clone 9.4 was then assayed on a set of peptides where the N-terminus was kept constant at position 102. It responded strongly to peptide 102–120, less well to peptide 102–118, and not to peptide 102–117, indicating that residue 118 was essential for stimulation. The epitope seen by this clone could therefore be defined by the seven amino acid sequence 112–118. Further analysis, however, suggested that the definition of an epitope could be rather more complicated. While the experiments described above showed that residues outside the sequence 112–118 were not essential for stimulation, peptide 112–118 failed to stimulate any proliferation, even at high concentrations (50 μM). The slightly longer peptide 111–118 did stimulate at the highest concentration, and this suggested that a minimum peptide length, as well as the appropriate amino acid sequence, was required for stimulation. The epitope seen by clone 9.4 could thus be defined either as residues 112–118 (as determined

with the overlapping sets of peptides) or as residues 111–118 (the shortest peptide to stimulate proliferation).

These experiments highlight one problem that continually arises in this kind of study: The designation of any peptide as stimulatory or nonstimulatory is an arbitrary one. In our experiments, any peptide that failed to stimulate proliferation at 50 μM was considered to be nonantigenic. However, a peptide that was nonantigenic at this concentration might perhaps stimulate, albeit weakly, at still higher concentrations. At what concentration does one stop? One example of this dilemma comes from experiments by Schwartz et al (50). The cytochrome *c* peptide 97–103 was found to be nonstimulatory at 10 μM for a particular T-cell clone, even though it spanned the two residues (99 and 103) known to be essential for stimulation. Theoretical considerations (discussed in the next section) suggested that this peptide should be able to adopt a conformation thought to be important for antigenicity. It was therefore tested on a second clone, apparently of the same specificity as the first, that responded to much lower doses of antigen. This second clone did respond to peptide 97–103 at the very high concentration of 100 μM; the epitope seen by this clone could thus be identified as the seven amino acid sequence 97–103. The definition of an epitope thus depends both on the antigen sensitivity of the T-cell clone in question and on arbitrarily imposed limits on the maximum concentration of peptide.

THE EFFECT OF PEPTIDE LENGTH ON ANTIGENIC POTENCY

The relationship between peptide length, secondary structure, and antigenicity has been analyzed in some detail by Schwartz and colleagues, working with Ia-restricted T-cell clones and hybridomas specific for an immunodominant epitope in the C-terminal cyanogen bromide fragment of cytochrome *c*. Experiments with synthetic peptide analogues of this fragment had suggested that residues 99 and 103 of moth cytochrome *c* were essential for T-cell stimulation (54, 55). However, peptide 97–103, which included both these residues, was unable to stimulate proliferation. The shortest peptide that could stimulate a T-cell response corresponded to the moth sequence 94–103, and maximum stimulation was only achieved with the much longer peptide 88–103. These results suggested that residues 88–98 were somehow important for the antigenic potency of these peptides.

One possibility was that these residues stabilized a particular secondary structure. Pincus et al (56) hypothesized that the lysine residue at position 99 had to adopt a particular orientation and that this was readily achieved only when the peptide was in an α helical conformation. They further suggested that residues 88–98 might be important because they stabilized this conformation. They therefore used a computer program to determine the preferred minimum energy conformations of various regions of this cytochrome *c* fragment. Residues 99–103 alone showed no obvious preference for any particular secondary structure, but the addition of residues 94–98 resulted in an α helical minimum energy conformation in a nonpolar environment. Schwartz et al (50) tested this hypothesis further by measuring the α helical content of stimulatory and nonstimulatory peptides by circular dichroism. In aqueous solution, none of the peptides had any detectable α helical content; in a relatively nonpolar solvent, however, many of the peptides adopted an α helical conformation. Moreover, there was a quantitative correlation between the tendency to form helical structures in solution and the ability to stimulate a T-cell response. From these results, Schwartz et al concluded that the antigenic potency of these cytochrome *c* peptides depended upon their tendency to adopt an α helical conformation in a nonpolar environment.

Watts et al (48) have also speculated that the antigenic potency of peptides might depend upon their ability to adope an α helical structure. Building on the idea that the antigen-Ia binding site might involve hydrophobic interactions (25, 41), they showed that while the linear sequence of the ovalbumin peptide 323–339 did not have a particularly obvious hydrophobic region, a hydrophobic surface could be generated by modeling the peptide as an α helix. DeLisi & Berzofsky (58) have suggested that secondary structures such as α helices are important for antigenicity precisely because they form stable amphipathic structures (see below).

The effects of increasing length on peptide antigenicity have now been demonstrated in a number of different systems. The idea that the addition of residues outside the epitope increases antigenic potency by stabilizing appropriate secondary structure is clearly seductive. The results are, however, compatible with the alternative hypothesis that while these residues are not essential for stimulation, they do in fact contact the T-cell receptor and/or the restricting Ia molecule and contribute directly to the affinity of the interaction between nominal antigen, Ia, and the T-cell receptor. The resolution of this question will probably have to wait until the structure of this ternary complex is elucidated.

MODELS FOR PREDICTING T-CELL EPITOPES

Studies on the specificity of B-cell responses to protein antigens have suggested that antibodies are directed predominantly against sites with certain characteristics, such as hydrophilicity and a high degree of mobility. In other words, these sites tend to be in highly exposed areas such as loops

and turns (57). It would obviously be useful to have similar guidelines for identifying sequences likely to act as T-cell epitopes. Two models have recently been proposed for the prediction of T-cell antigenic sites on proteins on the basis of primary sequence alone. Both have fulfilled the obvious criterion for a good model—the ability to make correct predictions. They differ completely, however, in the premises upon which these predictions are founded.

DeLisi & Berzofsky (58) have proposed that T-cell epitopes are likely to involve those sequences of a protein which can adopt stable amphipathic conformations. They suggest that such conformations might be induced or stabilized by hydrophobic interactions with structures such as Ia molecules on the antigen-presenting cell surface. Antigen processing experiments (24, 25) had shown that protein cleavage was not always necessary for effective presentation of antigen to T cells; that denaturation was sometimes sufficient. From this, Streicher and colleagues (25) argued that the function of antigen processing was to expose hydrophobic residues hidden inside the native protein, so that they could interact with Ia on the antigen presenting cell surface. They suggested that proteolytic cleavage was simply a rather drastic, but effective, method of unfolding proteins. DeLisi & Berzofsky used theoretical calculations to estimate the probability that a particular sequence would adopt an amphipathic conformation. They determined the periodicity of hydrophobic residues in the primary amino acid sequences of protein antigens and looked for segments with periodicity corresponding to ordered secondary structures such as α helices, or β-pleated sheets. They then asked whether these regions contained any of the known T-cell antigenic sites. At this time, 12 antigenic sites on 6 proteins (cytochrome *c*, insulin, lysozyme, myoglobin, ovalbumin, and influenza hemagglutinin) had been identified. Of these sites 10 fell within sequences which, on the basis of these calculations, could be modeled as amphipathic α helices.

This paper (58) made a prediction which, unknown to the authors, had already been verified in our laboratory. An analysis of the primary sequence of sperm whale myoglobin identified three blocks of sequence where the periodicity of hydrophobicity corresponded to that of an α helix. Two of these regions included known T-cell epitopes (51, 52). It was suggested that if there were another immunodominant site on this protein, then it might well fall within the third block of sequence (residues 69–81). We had in fact already identified an epitope within this region, contained within the 9-residue sequence 70–78 (53).

DeLisi & Berzofsky pointed out two constraints that applied to the interpretation of their model. In the first place, even though a protein might have several regions of amphipathic structure known to include T-cell epitopes, a single inbred strain should not be expected to respond to all these epitopes. Any one site might fail to stimulate a T-cell response in a given strain either because it could not associate with Ia, as proposed by the determinant selection model, or because of other constraints on the T-cell response. Secondly, while they argued strongly that T-cell epitopes were likely to have amphipathic properties, this did not mean that all sequences with amphipathic properties would be T-cell antigenic sites; it simply suggested that T-cell epitopes would most probably be located within such sequences.

Rothbard et al (59) have expressed reservations about this model, based principally on the fact that the number of epitopes analyzed in this study was small, while proteins with α helical structure in the native molecule were overrepresented. They adopted an empirical, rather than theoretical, approach to the identification of T-cell epitopes. They analyzed the primary amino acid sequences of the 28 T-cell antigenic sites known at the time, to see whether a pattern common to all these sequences could be found. Each residue was assigned to one of four categories: (*a*) hydrophobic; (*b*) charged; (*c*) polar; and (*d*) glycine and/or proline. Of these 28 antigenic sites, 27 were found to include one or more regions of sequence where a charged residue, or glycine, was followed by two hydrophobic residues. Since this pattern had been identified using a database of known T-cell epitopes, it clearly had to be tested for the ability to predict T-cell antigenic sites before it could be considered as characteristic of T-cell epitopes in general. Townsend et al (10) had demonstrated that short synthetic peptide analogues of influenza nucleoprotein could stimulate influenza-specific cytotoxic T cells. Rothbard and colleagues found that the cytotoxic response against influenza in one particular mouse strain was directed principally against the first 87 residues of this protein. This sequence was found to include three sites where a charged residue or glycine was followed by two hydrophobic residues. A peptide spanning one of these sites was synthesized and was shown to substitute in cytotoxic assays for the intact virus, the intact nucleoprotein, and for a deleted form of the nucleoprotein containing the first 87 residues (59).

The test for these models must surely lie in their ability to make correct predictions. Both models have correctly predicted a previously unknown T-cell antigenic site. One important difference between these models, however, is that while DeLisi & Berzofsky suggested an explanation for the importance of amphipathic structures, the three-residue template used by Rothbard et al was chosen on an empirical, rather than theoretical basis. The generality of these models can only be confirmed by the identification of new T-cell epitopes on a rather more diverse selection of proteins than have until now been studied.

SPECIFICITY OF THE INTERACTION BETWEEN Ia AND ANTIGEN

Although it is generally agreed that activation of the T cells studied in these systems requires the associative recognition of antigen and Ia by the T-cell receptor, not all the models for ternary complex formation require the physical association of antigen with the Ia molecule (reviewed by Schwartz: 2). Such an interaction is, however, central to the determinant selection hypothesis of immune responsiveness. In this section, we discuss experiments concerned with two questions: (*a*) Does nominal antigen bind to Ia on the antigen presenting cell surface; and (*b*) Do such associations show specificity that can be correlated with Ir gene effects?

The first experiments to address these questions looked at specific competition of T-cell activation by molecules that could bind to the restricting Ia molecule but were not stimulatory for the T-cell population in question. Werdelin (60) looked at the T-cell response in guinea pigs to two synthetic polymers, DNP-poly-L-lysine (DNP-PLL) and poly-$(Glu^{20}Lys^{80})_n$ (GL). Both DNP-PLL and GL elicited T-cell-proliferative responses in strain-2 guinea pigs, but DNP-PLL-specific T cells were not stimulated by GL. This T-cell response could, however, be blocked if the antigen-presenting cells were pulsed with nonstimulatory GL before being exposed to DNP-PLL. This result was compatible with the idea that both antigens were binding to the same site on the Ia molecules. There was, moreover, some evidence that this interaction was of relatively high affinity. Antigen presenting cells could be pulsed with GL, then washed, yet after 2 hr the ability of these cells to present DNP-PLL was still inhibited. This competition was apparently specific: GL did not block T-cell responses to ovalbumin, while another synthetic polymer, poly-L-arginine, did not block the response to DNP-PLL.

Rock & Benacerraf (61) demonstrated that this type of antigen competition was specific and that it operated at the level of the antigen presenting cell. The synthetic copolymer GAT could be recognized in association either with I-A^d or with I-A^b by appropriately restricted T-cell-hybridoma clones. Another synthetic polymer, GT, blocked the response of I-A^d restricted clones to GAT, while the response of I-A^b-restricted clones to GAT was unaffected. Rock & Benacerraf showed that $(b \times d)F_1$ antigen presenting cells cultured with GAT and GT were unable to stimulate I-A^d-restricted clones but could still stimulate I-A^b-restricted clones very effectively. They also showed that prepulsing the I-A^d-restricted T-cells with GT had no inhibitory effect on the ability to respond to GAT. These experiments argued against toxicity and nonspecific blocking mechanisms and supported the hypothesis that GAT and GT bound specifically to the same site on the I-A^d molecule.

The experiments of Werdelin and of Rock & Benacerraf were certainly compatible with a physical association between the synthetic polymers and Ia, but they did not demonstrate this directly. A number of groups have now used biophysical or biochemical techniques to look at interactions between antigen and Ia. The biophysical aspects of such interactions have been discussed in detail in the review by Watts & McConnell (62) in this volume, but we shall outline these experiments briefly again, in order to discuss the implications of the results for the specificity of T-cell activation.

Equilibrium dialysis has been used by two groups to show binding of antigenic peptides to the appropriate Ia molecules. Babbit et al (63) demonstrated that a fluorescently labeled hen egg lysozyme peptide HEL(46–61), which spans an immunodominant epitope on HEL seen by I-A^k-restricted T-cell hybridomas (41), was bound by detergent-solubilized I-A^k. The binding was weak but specific; the peptide did not bind to I-A^d. Similar experiments by Buus et al (64) showed that the chicken ovalbumin peptide OVA(323–339), which defines an ovalbumin epitope seen in association with I-A^d (37) bound weakly to purified I-A^d, but not to I-A^k or I-E^k.

In a subsequent paper, Babbit et al (65) showed that peptides which could competitively inhibit the response of a T-cell hybridoma to the HEL peptide 46-61 could also inhibit the binding of this peptide to purified Ia in equilibrium dialysis experiments. Allen et al (49) had previously analyzed the response of a T-cell hybridoma specific for HEL(52–61) to a series of substituted or truncated peptides spanning various parts of the HEL sequence 46-61; they had identified a series of these peptides that failed to stimulate proliferation. Babbit et al showed that some of these nonstimulatory peptides could block the cell response to HEL(46-61) and that these same nonstimulatory peptides could inhibit the binding of fluoresceinated HEL(46-61) to detergent-solubilized I-A^k in equilibrium dialysis experiments. The ability to block the T-cell response to HEL(46-61) correlated with the ability to inhibit the binding of fluoresceinated HEL(46-61) to I-A^k. This meant that the binding of HEL(46-61) to I-A^k in equilibrium dialysis experiments reflected a physiological interaction necessary for T-cell stimulation. It was particularly interesting that peptide Gln^{49} Phe^{56} 49-61, the sequence of which was identical with that of mouse lysozyme, inhibited the binding of HEL(46-61) to I-A^k in solution very effectively. Peptides that failed to block the T-cell response to HEL(46-61) also failed to inhibit the binding of HEL(46-61) to I-A^k. Fibrinopeptide B, which can stimulate a T-cell proliferative response restricted by I-A^k (66), was also unable to inhibit this interaction, a result that could suggest

two discreet binding sites for nominal antigen on the I-A^k molecule. Alternatively, HEL(46–61) and fibrinopeptide B might bind to the same site on I-A^k, but the interaction between fibrinopeptide B and I-A^k could be weak relative to that between HEL(46–61); if so, it should be possible to block the response of a fibrinopeptide B-specific, I-A^k-restricted T-cell clone with HEL(46–61). The evidence that Ia molecules have multiple binding sites for antigen is discussed in more detail in the next section.

Phillips et al (70) used another approach to demonstrate an interaction between antigen and Ia. They incubated radioiodinated, photoreactive beef insulin with antigen-presenting cells. After varying periods of incubation, the cells were exposed to light, which triggered the photoreactive roup, allowing it to bind covalently to adjacent molecules. When the cell membranes were solubilized and run out on gels, the radiolabel was found to be associated with two bands running at the appropriate size for Ia α and β chains. Moreover, the polypeptides running in these positions could be immunoprecipitated with haplotype-specific Ia alloantisera. These experiments showed that the photolabeled insulin bound specifically to Ia and not to other molecules on the antigen-presenting cell surface. However, this binding was not haplotype specific. The antigen bound not only to I-A^b and I-A^d, which are known to present insulin to T-cell hybridomas, but also to Ia molecules on cells from a panel of nonresponder haplotypes. In addition, it was pointed out that while photolabeled insulin coupled to cellular components could stimulate appropriate T-cell hybridomas, there was no evidence that the antigen responsible for this T-cell activation was still in the form seen in the gels. It was possible that these T cells recognized the antigen only after it had been cleaved from the Ia molecule.

Using Ia-containing planar membranes, Watts et al (71) were able to observe energy transfer between fluorescein-labeled OVA(323–339) and exas red-labeled I-A^d, indicating that the distance between donor and acceptor fluorophores was 40 Å or less. In contrast to the equilibrium dialysis experiments (63, 64), the peptide-Ia association responsible for this energy transfer was originally observed only in the presence of the appropriate T cell, specific for OVA(323–339) plus I-A^d. Further experiments by Watts & McConnell (72), however, showed that energy transfer between OVA(323–339) and I-A^d could be seen in the absence of T cells, if Ia and peptide were allowed to interact in serum-free conditions. First, they incubated either intact antigen presenting cells or a lysate of these cells with OVA(323–339) for 3 hr at 37°C and showed that membrane preparations from the lysed cells could stimulate T-cell activation almost as effectively as membranes prepared from the intact cells, even after exhaustive dialysis. This meant that the ability of pulsed cells to stimulate in these experiments could not be explained by the continual release of

antigen from intracellular pools but must instead be the result of a strong association between antigen and cell membrane components. They then pulsed phospholipid vesicles containing only lipid and I-A^d with fluoresceinated OVA(323–339), dialysed them, and showed that they too could stimulate the appropriate T-cell hybridoma. Stimulation was reduced if a proteolytic digest of hen egg lysozyme (pHEL) was added to OVA(323–339) at the time of the antigen pulse, but there was no inhibition if pHEL was added after the pulse, during the incubation of pulsed vesicles with the T cells. In addition, pHEL could not block the T-cell response to unpulsed vesicles in the presence of exogenous OVA(323–339). The association between OVA(323–339) and I-A^d was shown to be specific; this peptide did not bind to vesicles containing glycophorin instead of Ia.

These results suggested that the association between peptide and Ia was susceptible to competition by other protein fragments but was extremely stable once formed. They also demonstrated that this association could be stabilized by antigen-specific T cells, so that it was no longer vulnerable to competition. The energy transfer experiments were therefore repeated in serum-free conditions. Fluoresceinated OVA(323–339) was added in buffer with 1% fetal calf serum, as in the previously reported experiments (71), or in buffer without added serum, to planar membranes containing Texas-red-labeled I-A^d. In the absence of serum, an increase in Texas-red fluorescence was observed. The presence of 1% fetal calf serum or of unlabeled peptide reduced the fluorescence to background levels. While the initial association between peptide and Ia required quite high concentrations of peptide, the actual concentration of antigen required to stimulate T cells appeared to be extremely low. It was estimated that one T-cell could be activated by about 10^3 peptide molecules bound to Ia.

These results may explain why it has been so difficult to demonstrate inhibition of Ia-restricted T-cell responses to antigenic peptides by competition with nonstimulatory peptides known to bind to the Ia molecule in question (2). Watts & McConnell demonstrated, both in energy transfer and in antigen pulsing experiments, that T cells could stabilize antigen-Ia interactions in the presence of competing peptides or serum (72). In the experiments by Babbit et al (65), where T-cell responses were successfully blocked by a series of nonstimulatory peptides, the competing peptides were incubated with Ia for 30 min before the antigenic peptide and T cells were added.

The experiments discussed here show convincingly that antigen can bind specifically to Ia and not to other cell surface molecules and that this interaction between antigen and Ia reflects the associative recognition of these molecules by antigen-specific T cells. It is quite possible, however, that Ia-antigen binding sufficiently strong to be demonstrated in the

absence of T cells will be the exception rather than the rule. The HEL and OVA peptides used in these studies both span "immunodominant" epitopes. These epitopes are arguably immunodominant precisely because they form such a strong association with Ia. Schwartz (2) has suggested that the experiments of Werdelin (60) and Rock & Benacerraf (61) also belong in a special category; that the highly charged DNP-PLL, GAT, and GT peptides might bind nonspecifically to the cell surface and then bind specifically, but with relatively low binding affinities, to Ia.

It is too early to say whether these kinds of experiment support the determinant selection theory of immune responsiveness. They certainly confirm a central assumption of this theory, that T cells recognize a physical association between antigen and Ia. It seems reasonable to extrapolate from this that if an antigenic fragment is unable to associate with Ia, then it will not trigger an immune response. However, the converse is not necessarily true; peptides from protein antigens may associate with Ia, yet fail to stimulate an immune response because this would break self tolerance. There is clearly at least some specificity at the level of peptide-Ia binding. The hen egg lysozyme peptide HEL(46–61) bound to I-A^k but not to I-A^d (63), while the ovalbumin peptide OVA(323–339) bound to I-A^d but not to I-A^k or I-E^k (64). In both cases, these results reflected the specificity of the observed immunodominant T-cell response in the appropriate strains. The generality of these observations, however, has still to be tested. This should not be too difficult, since a reasonable number of epitopes seen preferentially in association with particular Ia molecules has now been defined. Whether there are multiple, distinct binding sites for antigen on any one Ia molecule has yet to be determined. Work from our laboratory has clearly demonstrated that there exist more than one "restriction site" on Ia molecules (67, 68); the possible location of such sites has been reviewed elsewhere (69). These results are not, however, incompatible with the idea that antigenic peptides bind to one particular region of the Ia molecule.

IMMUNODOMINANT REGIONS ON PROTEIN ANTIGENS STIMULATE HETEROGENEOUS T-CELL RESPONSES

A number of investigators have shown that the T-cell response to certain well-defined epitopes can be quite heterogeneous in fine specificity. Allen et al (49) showed that two I-A^k-restricted T-cell hybridomas, which both responded to a peptide spanning residues 52–61 of hen egg lysozyme, differed strikingly in their response to a panel of longer peptides. The response of clone 3A9 increased as the peptides became longer; clone

2A11, in contrast, responded strongly to the shortest stimulatory peptide (52–61), but progressively less well to the longer peptides. Another antigenic region of hen egg lysozyme has also been found to stimulate a heterogeneous T-cell response (44, 73). T-cell clones derived from H-2^b mice immunized with the middle cyanogen bromide fragment (residues 13–105) of hen egg lysozyme were all specific for epitopes within the tryptic peptide T11 (residues 74–96), but the T-cell clones showed three different patterns of reactivity to a panel of gallinaceous lysozymes. The clones were tested on shorter peptides and on peptides spanning residues 74–96, substituted to mimic the species variant lysozymes. All the clones responded to peptide 81–96 but differed in their responses to the substituted peptides. The differences in specificity could be attributed to sequence differences within the T11 region itself. To see whether this result was an isolated instance, B10.A mice were immunized with the HEL T11 peptide. The clones fell into two distinct groups. All the I-A^k-restricted clones responded to peptide 74–86, but not to peptide 81–96, while all the I-E^k-restricted clones responded to peptide 85–96. Again, clones within these two groups differed in fine specificity, as determined by their response to the panel of substituted peptides. Thus, within the immunodominant region 74–96, there are three distinct antigenic regions, spanning residues 74–86, 85–96, and 81–96, seen by T cells in association with I-A^k, I-E^k, and I-A^b, respectively, each of which can stimulate a heterogeneous T-cell response.

Our analysis of the T-cell response to sperm whale myoglobin in DBA/2 mice has revealed similar levels of complexity. We have focused on the response to two overlapping antigenic sites in the middle cyanogen bromide fragment (residues 56–131). The first site involves residues 111–118, and was identified by use of overlapping sets of peptides spanning residues 100–121 (53). The same set of peptides were used to characterize clones specific for the second site, an epitope first described by Berkower et al (35) centering on residue 109.

An analysis of clones recognizing the first antigenic site (residues 111–118) showed that sequences approaching the minimum size for T-cell activation (7–8 amino acids) could be recognized by T cells of at least three different specificities. We isolated a large number of clones reactive with residues 112–118, presented in association with I-E^d. These clones had indistinguishable patterns of response to the panel of peptides spanning residues 100–121. Most of them also responded equally well to sperm whale and horse myoglobins, which have lysine and arginine residues respectively at position 118. One clone, however, responded very poorly to horse myoglobin compared with sperm whale myoglobin, and this suggested that the 118-lysine to 118-arginine substitution had a deleterious

effect for the stimulation of this particular clone. In addition, this clone showed a pattern of cross-reaction on allogeneic stimulators, absent from all the other 112–118 reactive clones. A third clone, also I-E^d-restricted, recognized an epitope defined by residues 111–117. Thus the 8-residue sequence 111–118 was shown to include at least three distinct T-cell epitopes, all seen in association with I-E^d.

All the clones specific for the "109" epitope were I-A^d restricted, as observed for another H-2^d mouse strain by Berkower et al (43). We could, however, distinguish at least three reactivity patterns. One set of clones responded well to all the available peptides spanning residue 109; the longest peptides were the most stimulatory. These clones were stimulated peptides 108–118 and 102–117, indicating that the epitope lay within residues 108–117. The second group of clones also recognized an epitope within residues 108–117 but showed a completely different response to the synthetic peptides. They responded very strongly to peptide 102–118, but there was little or no response to the slightly longer peptides 102–120 and 100–121. A clone representing a third specificity was unable to respond either to peptide 102–117 or 108–118; it did, however, respond to peptides 106–121 and 102–118, indicating that the epitope seen by this clone lay somewhere within the 13-residue sequence 106–118. Similar results, using "109"-specific T-cell clones from B10.D2 mice, have been reported by Cease et al (52).

Three increasingly parsimonious models can be proposed to account for these results: (*a*) an antigenic peptide might be able to bind to multiple sites on any one Ia molecule; (*b*) the peptide might bind to only one site on Ia, but would be able to adopt multiple conformations; and (*c*) the peptide might bind in one conformation to one site on Ia. This third model is the one favored by Cease et al (52). They argue persuasively that their a, and all the other reports of heterogeneous T-cell responses to immunodominant antigenic sites, can be satisfactorily explained by a model where T-cells of multiple specificities are activated by antigen bound in a single conformation to one site on the Ia molecule. If this model is correct, then heterogeneity in the system must be caused entirely by the T-cell repertoire. It should be possible to resolve this question by antigenic competition studies using peptides that distinguish between clones of differing fine specificities.

WHY ARE THERE DIFFERENCES BETWEEN B-CELL AND T-CELL EPITOPES?

As discussed earlier in this review, the first indication that B cells and T cells might be recognizing different epitopes on the same antigen came

from the experiments of Gell & Benacerraf (12). The recognition that T cells immunized either with native or with denatured antigen could recognize the alternative form of the antigen, whereas B cells recognized only the form of antigen used for immunization together suggested a fundamental difference between B-cell and T-cell epitopes. The original hypothesis of Gell & Benacerraf was that antigenic structures seen by antibodies depended on tertiary configuration of the immunizing protein, whereas T cells (recognized by the authors as the DTH response) might recognize epitopes defined by primary amino acid sequence. Many other groups of investigators mentioned earlier in this review presented additional evidence that there was a fundamental difference between B- and T-cell epitopes. In addition to these experiments, Senyck et al (74) originally suggested that T- and B-cell epitopes occupied nonoverlapping areas on the immunizing molecule. These studies of the guinea pig responses to glucagon showed that the antibody response was directed primarily against the N-terminal portion of the molecule (residues 1–17), while all detectable T-cell reactivity appeared to be specific for the C-terminal section (residues 18–29). More recent experiments in the lysozyme model suggested that humoral and cellular responses were similarly directed against distinct portions of the molecule. Antibodies bound to an epitope involving the N-terminal residues, while the proliferative T-cell response was focused on the middle and C-terminal cyanogen bromide fragments (38, 39). In fact, the original idea of hapten and carrier determinants suggested that T cells and B cells recognized different epitopes. Haptens were defined as antigenic and were recognized by B cells, but they were not immunogenic. Carriers were portions of molecules required to allow the recognition and production of antihapten antibodies. These results suggest the possibility of a unifying theme for immune response, which was previously suggested by Abbas et al (75). This hypothesis suggests that B cells serve to present antigen to T cells, and this has been clearly documented (76–86). In addition, such antigen presentation should be more efficient if the antigen is recognized by the B cell, and this has also been demonstrated (75, 87, 88). The hypothesis we wish to examine may explain the fundamental difference between epitopes recognized by T cells and B cells. First, antigen-binding B cells recognize antigenic epitopes on the basis of tertiary configuration of the antigen via surface immunoglobulin. The antigen is then internalized and processed before presentation to T cells. The requirement for processing was demonstrated by metabolically inactivating B cells by fixation (75). Such hapten-binding B cells failed to present haptenated antigen if it was added subsequent to fixation or if such hapten-binding cells were fixed after a short (less than 4-hr) pulse with antigen. However, if sufficient time was allowed for antigen

recognition and processing (18 hr), such cells could subsequently be fixed and could function to present antigen to the antigen-specific T cells or hybridomas. Once internalized and processed, the tertiary configuration of the protein is disrupted and the carrier determinants, T-cell epitopes as defined in this review, can then be expressed on the surface of the B cell. They form the portion of the ternary complex required for T-cell activation in association with endogenous MHC class-II products present on the B-cell surface. An unanswered question is where the association of antigen and MHC class-II products occurs. Clearly, the results of Watts & McConnell presented earlier in this review demonstrate that it is possible to get appropriate presentation of T-cell epitopes by isolated I-region molecules nd to pulse fixed antigen-presenting cells with appropriate peptides for presentation. That such associations between processed antigen and I-region molecules can be formed with surface MHC class-II products does not necessarily mean that this is the usual route. It is more likely that I-region association with T-cell epitopes occurs intracellularly. This could, in fact, occur in the endocytic vesicle, which has been demonstrated to contain not only surface immunoglobulin, but also MHC class-II products from activated B cells (89, 90). This would assume that the MHC class-II product in some way escapes proteolytic degradation, whereas the nominal antigen undergoes appropriate cleavage and/or unfolding, a difficult scenario to accept. Alternatively, freshly synthesized MHC class-II products might join such endocytic vesicles following antigen degradation processes, and the association might be made prior to transport to the cell surface. In any event, this model clearly allows an antigen-specific B cell to present multiple T-cell epitopes to the immune system and thus to enhance its ability to be triggered in a specific manner. Such a model fulfills the known requirements for hapten carrier interaction, T-cell/B-cell MHC estrictions, and hapten-specific carrier-induced antibody responses. Further, it supports the notion that B-cell epitopes should, in most instances, be distinct from T-cell epitopes for the system to function efficiently.

SUMMARY

We have reviewed here studies using synthetic peptides to analyze some of the properties of T-cell epitopes. Several general conclusions can be drawn. First, T-cell epitopes can usually be defined by linear sequences of about seven amino acids. However, the observation that increasing peptide length often results in increased antigenic potency has suggested that antigenicity may crucially depend upon the ability of peptides to adopt appropriate secondary structures. Two models for the prediction of T-cell epitopes on the basis of primary sequence data alone were discussed.

Biophysical studies on the association of peptides with Ia molecules have shown that antigenic peptides bind directly to Ia; the evidence suggests that a binary association between Ia and peptide occurs in the absence of specific T-cells. Finally, a hypothesis to explain the observation that B-cells and T-cells generally recognize distinct epitopes on multideterminant antigens has been examined.

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(54) Title: A HUMAN T CELL REACTIVE FELINE PROTEIN (TRFP) ISOLATED FROM HOUSE DUST AND USES THEREFOR

TRFP CHAIN #1 PROTEIN SEQUENCE

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Cl Leader A          C I M K G A R V L V L L W A A L L L I W G G N C

Cl Leader B          A W R C S W K R M L D A A L P P C P T B A A T A D C

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Cl   E N A R I L K N C V D A K M T E E D K E N A L S L L D K I Y T S P L C

PRO. - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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(57) Abstract

A substantially pure, covalently linked human T cell reactive feline protein (TRFP) of approximately 40,000 MW has been isolated from vacuum bag extract obtained by affinity purification of house dust collected from several homes with cats; DNA encoding all or a portion of the TRFP or peptide; compositions containing such a protein or peptide or portions thereof; and antibodies reactive with the TRFP or peptide are disclosed. Also disclosed are recombinant TRFP or peptide; modified or mutated TRFP peptides; their use for diagnostic or therapeutic purposes.

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A HUMAN T CELL REACTIVE FELINE PROTEIN (TRFP) ISOLATED FROM HOUSE DUST AND USES THEREFOR

Description

Background

Genetically predisposed individuals, who make up about 10% of the population, become hypersensitized (allergic) to antigens from a variety of environmental sources to which they are exposed. Those antigens that can induce immediate and/or delayed types of hypersensitivity in people are called allergens. King, T.P., Adv. Immun., 23:77-105 (1976). The symptoms of hay fever, asthma and hives are forms of allergy which can be caused by a variety of allergens, such as products of grasses, trees, weeds, animal dander, insects, food, drugs and chemicals. The antibodies involved in allergy belong primarily to the IgE class of immunoglobulins. IgE binds to mast cells and basophils. Upon combination of a specific allergen with IgE bound to mast cells, the IgE is cross-linked on the cell surface, resulting in the physiological effects of IgE-antigen interaction. Degranulation results in release of, among other substances, histamine, heparin, a chemotactic factor for eosinophilic leukocytes and the leukotrienes, C4, D4 and E4, which cause prolonged constriction of bronchial smooth muscle cells. Hood, L.E. et al., Immunology, (2nd ed.), pp460-462, The Benjamin/Cumming Publishing Co., Inc. (1984). These released substances are the mediators which result in allergic symptoms caused by combination of IgE with a specific allergen. Through them, the effects of an allergen are manifested. Such effects may be systemic r local in nature, depending on the route by

which the antigen entered the body and the pattern of deposition of IgE and mast cells. Local manifestations generally occur on epithelial surfaces at the location at which the allergen entered the body. Systemic effects can include anaphylaxis (anaphylactic shock), which is the result of an IgE-basophil response to circulating (intravascular) antigen.

It has been estimated that there are approximately 10 million cat allergic individuals in the United States. Ohman, J.L., and Sundin, B., Clin. Rev. Allergy, 5:37-47 (1987). An allergen of particular concern for many people is the feline skin and salivary gland allergen of the domestic cat Felis domesticus allergen I (Fel d I), also referred to as allergen I, cat 1 and antigen 4. Fel d I has been described as an acidic non-covalently linked homodimer of approximately 39,000 molecular weight on size exclusion HPLC, and 17,000 under nonreducing conditions on gel electrophoresis. Chapman, M.D., et al. J. Immunology, 140(3):812-818 (1988). Chapman and co-workers also describe a single step procedure for the purification of Fel d I from crude house dust extract with a high Fel d I content (50 U/ml) using monoclonal antibody affinity chromatography. In addition, they determined the amino acid composition and partial amino acid sequence of Fel d I. Fel d I has also been described as a 35,000 molecular weight dimer of two noncovalently linked 18,000 molecular weight subunits, which occurs in three isoallergenic forms (pI 3.5 to 4.1). Ohman, J.L., et al., J. Allergy Clin. Immunol., 52:231 (1973); Ohman, J.L., et al., J. Immunol., 113:1668 (1974); Leiterman, K., and Ohman, J.L., J. Allergy Clin. Immunol., 74:147 (1984).

Exposure to cat allergen can occur as a result of exposure to the animal or contact with house dust which

contains cat allergens. These allergens have been examined in saliva, skin scrapings, cat wash, serum, salivary glands, cat hair, cat dandruff and house dust.

Despite the considerable attention allergic responses to cat allergens and cat allergens themselves have received, definition or characterization of the structure and components of the Fel d I allergen believed to be responsible for the adverse effects on cat-sensitive individuals is far from complete and current desensitization therapy involves treatment with a complex, ill-defined animal dander extract.

Summary of the Invention

The present invention relates to a substantially pure human T cell reactive feline protein, referred to as TRFP, of approximately 40,000 MW, isolated by affinity purification of house dust collected from several homes with cats; DNA encoding all or a portion of the TRFP or peptide; compositions containing such a protein or peptide or portions of the protein or peptide; and monoclonal antibodies reactive with the TRFP or peptide.

The present invention also relates to TRFP produced by recombinant techniques (recombinant TRFP) or a portion of recombinant TRFP or peptide. It further relates to TRFP, referred to as modified (or mutated) TRFP, in which the amino acid sequence differs from that of the naturally-occurring TRFP by an addition, deletion or substitution of at least one amino acid or the presence of another (non-amino acid) component. Such recombinant TRFP may be glycosylated or non-glycosylated, depending on the host cell used. As described herein it has been shown that natural TRFP is glycosylated (i.e., is carbohydrate-containing).

The present invention further relates to methods

of administering any of the forms of TRFP, (i.e., substantially pure TRFP, recombinant TRFP, modified TRFP) or a portion thereof, or a composition which includes a form of TRFP or a portion thereof, to reduce or prevent the adverse effects that exposure to cat allergens normally has on cat-allergic individuals (i.e., to desensitize individuals to cat allergens or block the effects of the allergens).

The present invention also relates to methods of diagnosing sensitivity to cat allergen and of predicting peptide(s) or amino acid sequence(s) useful in desensitization regimens. For example, as described herein, it has been shown that there are several peptides present within the TRFP which significantly stimulate T cells from cat allergic individuals. Such peptides have further been shown to affect lymphokine secretion profiles in different ways and in certain cases to anergize or tolerize T cells so that they no longer respond to TRFP. Such peptides can be administered in order to reduce or abolish an individual's allergic response to a cat allergen. Additionally, these peptides can be administered to cat-allergic individuals or used in ex vivo diagnostic tests to determine which one(s) cause the sensitivity. Those peptides determined to be applicable can be used selectively to desensitize a cat-sensitive individual. The term to "desensitize" is defined herein as to decrease the allergic-reactivity of a cat-sensitive individual to exposure to cats, cat dander or products thereof (to a level less than that which the cat-sensitive individual would otherwise experience).

The present invention further relates to a modified TRFP peptide or protein composed of a T cell epitope(s); compositions containing modified TRFP protein or portions

thereof; and to methods of administering the modified TRFP protein or portion thereof, alone or in combination, to reduce or prevent adverse effects that the unmodified, "naturally occurring" protein has on cat-sensitive individuals.

DNA of the present invention encoding all or a portion of the TRFP can be used as probes to locate equivalent sequences present in other species (e.g., goat, sheep, dog, rabbit, horse) that might be useful in a diagnostic and/or a therapeutic context.

Brief Description of the Drawings

Figure 1 is the DNA sequence and deduced amino acid sequence of TRFP, chain 1, leader A (underlined).

Figure 2 is the DNA sequence and deduced amino acid sequence of TRFP, chain 2, leader B (underlined).

Figure 3 is the DNA sequence and deduced amino acid sequence of TRFP, chain 2, long form (476 nucleotides).

Figure 4 is the DNA sequence and deduced amino acid sequence of TRFP, chain 2, short form (469 nucleotides).

Figure 5 is the DNA sequence and deduced amino acid sequence of TRFP, chain 2, truncated form (465 nucleotides).

Figure 6 is the protein sequence of TRFP, Chain 1, with leader A and leader B (top two lines). The deduced amino acid sequence of the leaders and for Chain 1 (C1) were obtained by sequencing cDNA and the protein sequence for Chain 1 (PRO) was determined by protein sequencing methods, with amino acid numbering based upon the first amino acid determined by protein sequencing methods. The presumed initiator methionine in each leader sequence is in bold type. (-) symbolizes complete agreement or identity with the amino acid residue listed above the (-).

Figure 7 is the protein sequence of TRFP, Chain 2 with the leader sequence. The deduced amino acid sequence of the leader and for Chain 2 (C2) were obtained by sequencing cDNA. The protein sequence for Chain 2 (PRO) was determined by protein sequencing methods, with amino acid numbering based upon the first amino acid and polymorphism detected by protein sequencing methods noted. C2L: chain 2 long (92 amino acids); C2S: chain 2 short (90 amino acids); C2ST chain 2 short truncated (80 amino acids). The presumed initiator methionine in the leader sequence is in bold type and the potential N-glycosylation site is underlined. (-) symbolizes complete agreement or identity with the amino acid residue listed above the (-).

Figure 8 shows the results of SDS/PAGE Western immunoblot analysis of affinity purified TRFP under reduced conditions probed with affinity purified rabbit anti-peptide antibodies (anti-Fel 2, Fel 4, and Fel 18); monoclonal anti-Fel d I antibody (6F9) and pooled cat allergic human serum IgE. The anti-Fel 2, Fel 4 antisera are specific for chain 1. The anti-Fel 18 antiserum is specific for chain 2.

Figure 9 is a graphic representation of the secondary T cell response of peripheral blood lymphocytes from patient 131 stimulated with various antigens and peptides.

Detailed Description of the Invention

As described herein, TRFP has been isolated and purified by affinity purification of vacuum cleaner bag house dust collected from several homes with cats. The work described herein has resulted in isolation and purification of a TRFP protein; determination of the nucleotide sequence encoding TRFP and the amino acid

sequence of TRFP (Figures 1-7); demonstration that TRFP is composed of two covalently linked peptide chains (designated chain 1 and 2); identification and isolation of T cell reactive peptides or amino acid sequences present in the TRFP protein; and characterization of TRFP. It has also resulted in cloning and expression of TRFP in E. coli and characterization of the resulting recombinant TRFP proteins. As described in Example 4, cDNA clones encoding all or part of TRFP chain 1 or chain 2 have been expressed in E. coli as recombinant fusion proteins. Of note is the finding that chain 1 of the two-chain TRFP protein has two alternative leader sequences and that chain 2 has two major forms (designated as long and short).

A monoclonal antibody reactive with Felis domesticus allergen I, known as Fel d I, was used to isolate a single protein from a vacuum cleaner bag preparation. The affinity purified T cell reactive protein isolated in this manner is referred to as human T cell reactive feline protein (human TRFP). TRFP has been shown to have biological activity (human IgE binding ability) and to possess cross reactivity with rabbit anti-Fel d I antisera. The term "allergenic" as used herein in referring to peptides or proteins of the present invention refers to those peptides or proteins which bind IgE and/or stimulate T cells.

In addition to determining the amino acid sequence of chains 1 and 2 of the TRFP, a Fel d I protein preparation provided by Martin Chapman was analyzed and the protein was isolated and sequenced. Comparison of the amino acid sequence of the affinity purified TRFP with that of the published Fel d I protein sequence showed that there is a high degree of homology between the first 33 amino acid sequences at the amino terminus

of Fel d I and chain 1 of TRFP.

The following is a description of the methods by which a single protein composed of two covalently linked chains was isolated from house dust, as well as a description of approaches used to identify and isolate DNA encoding the TRFP. Furthermore, a description of methods used to generate recombinant TRFP chains 1 and 2 are also presented. Additionally, human T cell epitopes from the TRFP protein have been identified and are described herein.

Isolation of a single protein from a vacuum cleaner bag preparation

A protein preparation was extracted from the contents of vacuum cleaner bags by a method based on that of M.D. Chapman and co-workers. Chapman, M.D., et al, J. Immunol., 140(3): 812-818 (1988). Monoclonal antibody reactive with Fel d I, produced by Chapman and co-workers, was used to identify a protein in the preparation. de Groot H. et al., J. Allergy Clin. Immunol., 82:778-786 (1988). Selected monoclonal antibodies (designated 1G9 and 6F9) that recognize Fel d I native protein were used to affinity purify a protein, which is referred to as human T cell reactive feline protein(TRFP) (also referred to as VCB or vacuum cleaner bag protein) from a house dust sample. This was carried out, using known techniques, by producing the desired monoclonal antibody, isolating it in large quantities from ascites and immobilizing it on Sepharose 4B (Pharmacia). The protein preparation was extracted from vacuum cleaner bags of house dust obtained from several homes with cats. Aqueous vacuum cleaner bag extract was first subjected to gel filtration and decolorization and, subsequently, affinity chromatography purification.

Aqueous vacuum cleaner bag extract was passed over the monoclonal antibody-containing column and a protein species was eluted. The protein isolated in this manner was shown, using both Western blot and ELISA techniques, to bind human IgE, thus demonstrating that TRFP possesses allergenic activity. The affinity purified TRFP was subjected to a number of protein chemical procedures to derive primary amino sequence data. The sequences derived from TRFP are illustrated in Figures 6 and 7. The methods used in the protein sequence analysis are further described in Example 1. Under non-reducing conditions, Western blot analysis demonstrated the existence of a 40kD and a 20kD species, whereas a 10-18 kD and a 5kD species was detected under reducing conditions (Figure 8).

The 5 kD band interacts with affinity purified anti-peptide antisera raised against peptides derived from chain 1 protein sequence (anti-Fel 2 and anti-Fel 4), whereas the 10-18 kD band interacts with antipeptide antiserum raised against peptide derived from chain 2 protein sequence (anti-Fel 18). Hence, the 5 kD band and the 10-18 kD band are derived from the TRFP chain 1 and the chain 2, respectively. TRFP can exist as an aggregated form, as demonstrated by the approximately 40 kD molecular weight of the affinity purified TRFP (may be a dimer of the chain 1 and chain 2 heterodimer) and the approximately 130 kD species detected in gel filtration prior to affinity purification.

Identification of clones containing DNA inserts encoding the human T cell reactive feline protein (TRFP)

Protein chemical analysis of affinity purified TRFP led to the determination that TRFP is composed of two covalently linked peptide chains (designated chain 1 and

2; see Example 1 for details). Furthermore, peptide sequence analysis led to the determination of considerable primary sequence data for both chain 1 (70 amino acids; see Figure 6) and chain 2 (83 amino acids; see Figure 7). The amino acid sequence data was used to devise various cloning strategies to enable the cloning and complete nucleotide sequence determination of cDNAs and genomic clones encoding the TRFP chains 1 and 2 (details provided in Examples 2 and 3).

In order to determine the best tissue source(s) to isolate mRNA for the cloning of TRFP, various cat tissues were examined by ELISA techniques using monoclonal antibodies (directed against Fel d I). It was determined that the several salivary glands and skin contain significant levels of TRFP, and thus, provide a valuable source from which to clone cDNA sequences encoding the TRFP (see Table 1, in which --- indicates that an analysis was not done).

Table 1

Micrograms TRFP/gram tissue

Tissue	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5
Parotid	1.03	1.41	0.81	0.32	0.30
Mandibular	0.41	2.39	7.50	2.50	4.66
Sublingual	0.77	----	0.50	3.18	3.82
Zygomatic	----	----	2.07	8.50	10.9
Molar	----	----	7.58	1.47	25.00
Palate	----	----	1.03	0.53	0.77
Washed Skin	5.80	2.30	----	----	----

Several approaches can be used to clone the TRFP encoding cDNAs including oligonucleotide screening of a λ gt10 or λ gt11 cDNA library. Alternatively, anti-peptide antisera reactive with TRFP amino acid sequences (see

Figures 6 and 7) can be used to screen a gt11 library using standard methods. Young, R.A. and R.W. Davis, Proceedings of the National Academy of Sciences, USA, 80: 1194-1198 (1983). DNA can be isolated from reactive clones and sequenced using the method of Sanger and co-workers. Sanger, F. et al Proc. Natl. Acad. Sci., USA 74: 5463 (1977).

Another approach to clone DNA encoding the human T cell reactive feline protein is to use polymerase chain reaction (PCR) technology to amplify and clone DNA from cat salivary gland mRNA or genomic DNA. Mixed oligonucleotide primers (forward and reverse) were deduced from the known amino acid sequence and used in a PCR to produce a partial cDNA clone. This strategy, termed mixed oligonucleotide primed amplification of cDNA (MOPAC), is described by Lee and co-workers (Lee et al., Science, 239: 1288-1291 (1988)). MOPAC (and derived methods) have been used to isolate both partial and full-length cDNAs encoding the TRFP chains 1 and 2 (Detailed in Example 2 and the nucleotide sequences illustrated in Figures 1-6). The PCR derived cDNA clones were used as ^{32}P-labelled probes to screen a cat genomic EMBL4 library, as described in Example 3.

As a result of the work described herein, cDNAs and genomic clones encoding chain 1 and chain 2 of TRFP have been cloned, isolated and sequenced; the encoded amino acid sequences of the protein has been deduced; and peptides derived from TRFP have been identified and isolated using known methods. The complete nucleotide sequences encoding both TRFP chains are shown in Figures 1-5. The hybridization pattern of individual genomic clones verified that the chain 1 and chain 2 cDNAs are products of different genes. Northern blot analysis of the cat salivary gland RNA also demonstrated the presence

of the two separate mRNAs. Sequencing of the genomic clones confirmed the hybridization results. As described in Example 2, individual full-length PCR generated chain 1 clones were shown to have two different sequences at their 5' ends, suggesting that chain 1 has two alternative leader sequences. This was confirmed by the DNA sequence analysis of the chain 1 genomic clone, which demonstrated that the single chain 1 gene has both alternative leader sequences closely linked at the 5' end of the structural gene (see Figures 1, 2 and 6).

As described in Example 4, cDNA clones encoding all or a fragment of TRFP chain 1 or chain 2 were subcloned into E. coli expression vectors and the expressed recombinant TRFP proteins examined. Western blot analysis using rabbit anti-peptide antisera directed against either chain 1 sequences or chain 2 sequences demonstrated appropriate binding specificity.

Uses of the subject human T cell reactive feline protein (TRFP) and DNA encoding same

The materials resulting from the work described herein, as well as compositions containing these materials, can be used in methods of diagnosing, treating and preventing cat allergy. In addition, the cDNA (or the mRNA from which it was reverse transcribed) can be used to identify similar sequences in other species (e.g., sheep, goat, dog, rabbit, horse) and, thus, to identify or "pull out" sequences that have sufficient homology to hybridize to the TRFP cDNA. Such sequences from other species might encode proteins useful in treating allergies to these animals in people. This can be carried out, for example, under conditions of low stringency and those sequences having sufficient homology (generally greater than 40%) can be selected for further

assessment using the method described herein. Alternatively, high stringency conditions can be used. In this manner, DNA of the present invention can be used to identify, in other types of mammals (e.g., dog, rabbit, sheep, goat, horse), sequences encoding peptides having amino acid sequences similar to that of the TRFP. This can be done by hybridization or PCR cloning methods. Thus, the present invention includes not only the TRFP or peptide encoded by the present DNA sequences, but also other TRFP-like proteins or allergenic peptides encoded by DNA which hybridizes to DNA of the present invention.

The TRFP peptide encoded by the cDNA of the present invention can be used, for example, as "purified" TRFP, in a composition to treat cat-allergic individuals, in a method to diagnose cat allergy, or in the standardization of allergen extracts which are key reagents for the diagnosis and treatment of cat allergy. Through use of the protein of the present invention, allergen preparations of consistent, well-defined composition and biological activity can be made and administered for therapeutic purposes (e.g., to modify the allergic response of a cat-sensitive individual to cat allergies). Such a protein or peptide (or modified version thereof, such as is described below) may, for example, modify B-cell response to cat allergen, T-cell response to cat allergen or both responses. Purified TRFP or peptide thereof can also be used to study the mechanism of immunotherapy of cat allergy and to design modified derivatives or analogues that are more useful in immunotherapy than are the unmodified ("naturally-occurring") protein or peptide.

Work by others has shown that high doses of allergens during immunotherapy treatment generally produce the best results (i.e., best symptom relief). However, many

people are unable to tolerate large doses of allergens because of adverse reactions to the allergens.

The present invention enables the production of therapeutic treatments for cat allergic individuals which will possess similar or improved efficacy to that of current allergen immunotherapy without the adverse reactions normally associated with this form of therapy. Improved therapy could derive from use of modified naturally occurring TRFP or peptide expression products of the TRFP genes identified herein or appropriate modifications (mutations) thereof, or peptides derived from the structure of TRFP or modifications thereof.

For example, the naturally occurring TRFP or peptide can be modified using the polyethylene glycol method of A. Sehon and coworkers or in other ways which reduce the IgE reactivity of the natural allergen and thereby decrease its adverse reaction potential.

Alternatively, the TRFP cDNAs defined herein, or portions thereof, can be expressed in appropriate systems to produce protein(s) with strong therapeutic activity, but greatly reduced ability to bind to IgE and thereby produce adverse reactions. To facilitate this, it is possible to add reporter group(s) to the chain 1 and/or 2 polypeptide backbone as an aid to efficient purification. One such reporter group is poly-histidine, which has been effectively used to purify recombinant proteins on immobilized metal ion affinity chromatography (Hochuli, E. et al., Bio/Technology 6:1321-1325 (1988)). Specific cleavage sites can be introduced between the reporter group and the chain 1 and 2 polypeptide sequences, and cleavage at these sites can facilitate the isolation of TRFP chains or fragments free of irrelevant sequences. Another example of the modification of the TRFP chains 1 and 2 is the substitution of cysteine residues with

another amino acid residue such as serine (or any other residue) to reduce disulfide complexes.

Site-directed mutagenesis of the TRFP cDNAs can also be used to modify the chain 1 and 2 structures. Such methods may involve PCR (Ho et al., Gene 77:51-59 (1989)) or total synthesis of mutated genes (Hostomsky, Z. et al., Biochem. Biophys. Res. Comm. 161:1056-1063 (1989)) since the two chains are each composed of coding sequences <400bp. To enhance bacterial expression, the aforementioned methods can be used in conjunction with other procedures to change the mammalian codons in the constructs to ones preferentially used in E. coli.

Other modifications of the TRFP genes may include the construction of gene chimeras, where chains 1 and 2, or parts thereof, may be linked to form a single contiguous chain. For example, all or a portion of chain 1 may be linked with all or a portion of chain 2 cDNA and the resulting chimera may be produced as a recombinant hybrid (Horton et al., Gene 77:61-68 (1989)). It is also possible to construct multiple joined genes to promote stability of the expressed product or to enhance its therapeutic potential (Shen et al., Proc. Natl. Acad. Sci. USA 81:4627-4631 (1984)).

The work described herein has further resulted in identification of certain areas of the TRFP protein which contain peptide epitope sequences which powerfully stimulate T cells from cat allergic individuals. It is believed that these T cell epitopes are intimately involved in initiation and perpetuation of the immune responses to cat allergen(s) which are responsible for the clinical symptoms of cat allergy. Such epitopes from the natural cat allergen(s) are believed to trigger early events in the allergic cascade at the level of the T helper cell by binding to an appropriate HLA molecule

on the surface of an antigen presenting cell and stimulating the relevant T cell subpopulation. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions and the recruitment of additional immune cells to the site and activation of the B cell cascade leading to production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events, at the level of the T helper cell, by the nature of the lymphokines secreted. Exogenously administered T cell epitope peptides may thus influence the development or perpetuation of the allergic response to cat allergens and produce therapeutic benefit in cat allergic individuals. Thus, exposure of cat allergic patients to T cell epitope peptides identified as described herein may tolerize or anergize appropriate T cell subpopulations so that they no longer respond to cat allergen(s) and do not participate in mounting the immune response to such exposure. Alternatively, administration of the T cell epitope peptides may drive the lymphokine secretion profile in a different direction than is the case with exposure to natural allergen(s) (e.g. decreased IL-4 and/or increased IL-2), resulting in a reduction of local inflammatory events and/or a beneficial change in the antibody secretion profile. Alternatively, exposure to T cell epitope peptides may cause T cell subpopulations which normally participate in the response to cat allergen(s) to be drawn away from the site(s) of normal exposure to the allergen (nasal mucosa, skin, lung) towards the site(s) of therapeutic administration of the peptides. This redistribution of T cell subpopulations could ameliorate or reduce the ability of an individual's immune system to mount the usual immune

response to the allergen(s) at the site of normal exposure, leading to a diminution in allergic symptoms.

Peptides of varying sizes from within the structure of TRFP have been synthesized and purified by conventional techniques and examined for their biological effects on T cell lines and clones obtained from human cat allergic individuals. The methodologies used are described in Examples 5, 6 and 7.

Peptides from within the structure of TRFP have been shown to stimulate a proliferative response in TRFP primed T cell cultures. Indeed in certain cases (Figure 9), the proliferative response obtained with the peptides can substantially exceed that obtainable with TRFP or cat skin test allergen preparations.

It is also apparent (Tables 2 and 3) that certain areas of the TRFP sequence have weak T cell stimulatory activity (e.g. Fel 4-3, Fel 28-1); certain other areas have powerful activity (e.g. Fel 8-3, Fel 14). This range of activities may derive from purely primary sequence differences or from physicochemical differences induced by primary sequence changes. Key peptide epitopes from within the structure of TRFP which are highly reactive with T cells from cat allergic patients can be identified using the subject disclosure and known techniques.

Furthermore, it has been demonstrated that exposure of T cells to epitope peptides <u>in vitro</u> under conditions which simulate therapeutic treatment regimens can suppress a subsequent response to the allergen (TRFP) to a greater extent than that obtained with the allergen alone (Example 6, Table 4). This data points to the clear opportunity of selecting epitope peptides identified by the current work and applying them in treatment paradigms in cat allergic patients designed to

suppress their response to cat allergen exposure.

In addition, it has been shown that exposure of T cells from cat allergic individuals to different epitope peptides from TRFP can produce distinctly different lymphokine secretion profiles (Example 7, Table 5). It is thus possible using the current invention to select for therapeutic application epitope peptides which drive a lymphokine secretion profile consistent with a therapeutically beneficial response upon treatment of cat allergic patients.

Administration of a TRFP protein or peptide of the present invention, which can be substantially pure TRFP, recombinant TRFP, modified TRFP, alone or in combination, to an individual to be desensitized can be carried out using known techniques. A peptide or combination of two or more different peptides can be administered to an individual in a composition which also includes, for example, an appropriate buffer, a carrier and/or an adjuvant. Such compositions will generally be administered by injection, inhalation, transdermal application, intranasal application, oral application or rectal administration. Using the structural information now available, it is possible to design a TRFP or peptide that, when administered to a cat-allergic individual in sufficient quantities, will modify the individual's allergic response to cat allergen. This can be done, for example, by examining the structures of the TRFP, producing peptides to be examined for their ability to influence B-cell and/or T-cell responses in cat-allergic individuals and selecting appropriate epitopes recognized by the cells. Synthetic amino acid sequences which mimic those of the epitopes and which are capable of down regulating allergic responses to cat allergen can be produced. The protein, peptide or antibodies of the

present invention can also be used, in known methods, for detecting and diagnosing cat allergy. For example, blood obtained from an individual to be assessed for sensitivity to cat allergen is combined with an isolated peptide of TRFP, under conditions appropriate for binding of components (e.g., antibodies, T cells, B cells) in the blood with the peptide. Subsequently, the extent to which such binding occurs is determined, using direct (e.g., determination of T cell activation) or indirect methods.

It is now also possible to design an agent or a drug capable of blocking or inhibiting the ability of cat allergens to induce an allergic reaction in cat-allergic individuals. Such agents can be designed, for example, in such a manner that they would bind to relevant anti-cat allergen IgEs, thus preventing IgE-allergen binding and subsequent mast cell degranulation. Alternatively, it is possible to design agents which bind to cellular components of the immune system, resulting in suppression or desensitization of the allergic response to cat allergen. A non-restrictive example of this is the use of appropriate B- and T-cell epitope peptides, or modifications thereof, based on the cDNA/protein structure of the human T cell reactive feline protein of the present invention to suppress the allergic response to cat allergen. This can be carried out by defining the structures of B- and T-cell epitope peptides which affect B- and T-cell function in <u>in vitro</u> studies with blood cells from cat-sensitive individuals. This procedure is described in detail in Example 5.

It is also possible to modify epitopes of the TRFP, to combine epitopes, or to do both, for such purposes as enhancing therapeutic or preventive efficacy, stability (e.g., length of time for which they can be stored), and

resistance to degradation in the body of TRFP peptides. For example, the amino acid residues essential to epitope function can be determined using known techniques (e.g., substitution of each residue and determination of presence or absence of T cell reactivity). Those residues shown to be essential can be modified (e.g., replaced by another amino acid whose presence is shown to enhance T cell reactivity), as can those which are not required for T cell reactivity (e.g., by being replaced by another amino acid whose incorporation enhances T cell reactivity).

Two or more TRFP epitopes can also be combined in order to enhance, for example, therapeutic effectiveness. For example, the amino acid sequences of two epitopes present within the first 30 N-terminal amino acids can be produced and joined by a linker. The linker by which the epitopes are joined can be any non-epitope amino acid sequence or other appropriate linking or joining agent. The epitopes joined in this manner can be from the same chain of the TRFP or from different TRFP chains (e.g., one from chain 1 and one from chain 2). The resulting two-epitope construct can be used in treating cat-sensitive individuals. Alternatively, an epitope (or epitopes) present in the first chain of the TRFP and one (or more) present in the second chain can be joined to produce a construct which has greater therapeutic effectiveness than a single epitope peptide. Additionally, individual peptides can be physically mixed and administered as a therapeutant.

DNA to be used in any embodiment of this invention can be cDNA obtained as described herein or, alternatively, can be any oligodeoxynucleotide sequence that codes for all or a portion of the amino acid sequence represented in Figures 1-7, or the functional equivalent

thereof. Such oligodeoxynucleotide sequences can be produced chemically or mechanically, using known techniques. A functional equivalent of an oligonucleotide sequence is one that is capable of hybridizing to a complementary oligonucleotide sequence to which the sequence (or corresponding sequence portions) of Figures 1-7 hybridizes and/or that encodes a product (e.g., a polypeptide or peptide) having the same functional characteristics of the product encoded by the sequence (or corresponding sequence portion) of Figures 1-7. Whether a functional equivalent must meet one or both criteria will depend on its use (e.g., if it is to be used only as an oligoprobe, it need meet only the first criterion and if it is to be used to produce an allergen, it need meet only the second criterion).

The structural information that is available or can be deduced from the amino acid sequences of Figures 1-7 (e.g., DNA, protein/peptide sequences), can also be used to identify or define T cell epitope peptides and/or B cell epitope peptides which are of importance in cat allergic reactions and to elucidate the mediators or mechanisms (e.g., interleukin-2, interleukin-4, gamma interferon) by which these reactions occur. This knowledge should make it possible to design peptide-based cat allergen therapeutic agents or drugs which can be used to modulate these responses.

The present invention will now be further illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLE 1. Isolation and Protein Sequence Analysis of the T Cell Reactive Feline Protein (TRFP)

Monoclonal affinity purification of a T cell reactive feline protein from house dust extract

A house dust sample collected from several homes with cats was used to isolate and purify TRFP. Monoclonal antibodies 1G9 or 6F9 were coupled to Sepharose 4B and used for the purification according to a published protocol. Chapman, M.D., et al., J. Immunology, 140(3):812-818, (1988). The purified TRFP was decolorized by loading it on a Phenyl-Sepharose column (Pharmacia) with 4N NaCl, then eluted with 2M and 1M NaCl. Decolorized TRFP was recovered by dialyzing the 2M and 1M salt eluates against distilled water and lyophilized. Decolorization was also carried out by passing the house dust extract through a Sephacryl 200 column (Pharmacia) before the affinity purification.

Preparation of TRFP peptides

Affinity purified TRFP was first reduced with dithiothreitol and then alkylated with 4-vinyl pyridine. After desalting with a Sephadex G10 column, the reduced and alkylated TRFP was cleaved chemically with cyanogen bromide (CNBr) or enzymatically with one of the following enzymes: endoproteinase Glu-C (Boehringer Mannheim), endoproteinase Asp-N (Boehringer Mannheim), endoproteinase Lys-C (Boehringer Mannheim). The affinity purified TRFP was also digested by trypsin (Worthington) without reduction and alkylation. The digestion products were separated on an Aquaport RP300 column (C8) with acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). The individual peaks were subjected to the protein sequencer.

Peptide and protein sequence analysis

An Applied Biosystems Model 477A gas phase sequencer with on-line phenylthiohydantoin (PTH) amino acid analysis (Model 120A) was used. A modification of extraction program, multiple butylchloride extractions, was used to improve the amino acid recovery. O-phthalaldehyde was used in blocking of primary amines when proline was located at the amino terminus. Brauer, A.W., et al., Anal. Biochemistry, 137:134-142, (1984). In situ alkylation was performed by using the non-nucleophilic reductant, tributylphosphine with concomitant alkylation by 4-vinyl pyridine in ethylmorpholine buffer. Andrews, P.C. and Dixon, J.E., Anal. Biochemistry, 161:524-528 (1987). The N-terminal protein sequence analysis of the intact TRFP protein revealed that there is one major amino acid sequence and several minor amino acid sequences. The major sequence (chain 1 in Figure 6) corresponds to the published Fel d I N-terminal 33 amino acid residues with two significant differences. Chapman, M.D., et al., J. Immunology, 140(3):812-818, (1988). The most prevalent minor sequence (at 55% the level of the major sequence) was designated as chain 2 (Figure 7). The other minor sequences were various N-terminal truncated forms of chain 2. Since the 4th residue of chain 1 and the 7th residue of chain 2 were proline, o-phthaladehyde was applied before the 4th cycle or the 7th cycle of Edman degradation to block out chain 2 or chain 1 sequences, respectively. The protein sequence information of chain 1 (68 N-terminal amino acid residues) and chain 2 (58 N-terminal amino acid residues) was further enhanced by an additional OPA blocking before cycle 32 and cycle 37 for chain 1 and chain 2, respectively. The protein sequences were confirmed and expanded by sequence analysis of

enzymic and chemical digested peptides. Time dependent in situ CNBr digestion of TRFP on the sequencer glass filter disk could provide additional protein sequence information. Simpson, R. J. and Nice, E. C., Biochem. International, 8:787-791 (1984). Prior to the in situ CNBr digestion five sequencer cycles were performed and then the protein sample was treated with acetic anhydride to block all amino groups. These steps removed the N-terminal five residues from both chains and blocked the amino groups of the next residue from both chains and any other peptide in the sample. After 5 hours of in situ CNBr digestion, one major peptide sequence, CB-1, and three minor peptides which had 60% (CB-2), 38% (CB-3), and 12% (CB-4) signal levels of the major peptide sequence were identified. CB-1 started from residue 43 of chain 2 and extended the N-terminal sequence of chain 2 to 68 residues. CB-2 was identical to a purified CNBr peptide sequence of chain 2 (75-80). CB-3 corresponded to the peptide sequence 65-68 of short form chain 2. CB-4 corresponded to the peptide sequence 50-66 of chain 1. A tryptic peptide TRYP-1 (short form chain 2, 58-80) connected the 68 residue N-terminal peptide with an endopeptidase Asp-N generated peptide, D-10 (chain 2, 72-83), and extended the chain 2 to 83 amino acid residues. An endopeptidase Lys-C generated peptide, K13 (chain 1, 64-70), extended chain 1 to 70 amino acid residues. Other enzymic and CNBr generated peptides confirmed the N-terminal sequences of chain 1 and chain 2. The sequences of a short tryptic peptide, TRYP-2 (long form chain 2, 58-69), and an endopeptidase Asp-N peptide, D-10, revealed that there was sequence polymorphism in chain 2 residues 65 to 72. In summary, the primary amino acid sequence of TRFP chains 1 and 2 derived by protein sequencing methods is presented in Figures 6 and 7.

There is a potential N-glycosylation site present in the cDNA deduced amino acid sequence, Asn_{33}-Ala_{34}-Thr_{35}. The protein sequence analysis identifies the Ala_{34} and Thr_{35} of chain 2; however, nothing can be identified at the position 33. It suggests that post-translation modification occurs at Asn_{35} of chain 2 and the modification is stable to the trifluro acetic acid treatment during protein sequencing. The hypothesis was confirmed by treating TRFP with N-peptidase F (Boehringer Mannheim) which reduced the size of chain 2 to 7-12 kD in SDS-PAGE/Western immunoblot. Moreover, both chains can be modified by ß-elimination which implies they may have O-linked glycosylation. The two chains are covalently linked together (approximately 20kD) through disulfide bond(s).

EXAMPLE 2. Cloning of cDNAs Encoding Chains 1 and 2 of the Human T cell Reactive Feline Protein (TRFP)

MOPAC (and derived methods) have been used to isolate both partial and full-lengthcDNAs encoding the TRFP chains 1 and 2. The PCR methods used are described in detail below.

Cloning of TRFP chain 1 cDNAs

First strand cDNA synthesis was performed with 1 µg of poly A plus RNA isolated from a pooled sample of cat parotid and mandibular glands using oligo dT primer.

MOPAC PCR amplification (Lee et al, Science 239:1288-1291(1988)) of an internal portion of chain 1 was carried out using a sense/antisense pair of degenerate oligonucleotide primers encoding amino acids 1-6 and 50-54 of chain 1, respectively (see below). These oligonucleotides (primers 1 and 2) were used with a

Perkin Elmer/Cetus PCR kit to amplify an aliquot of the above cDNA using the following cycles:

94° C 1 min. (denaturation)
45° C 1 min. 30 sec. (annealing)
72° C 1 min. (polymerization) 5 Cycles

94° C 1 min. (denaturation)
55° C 1 min. 30 sec. (annealing
72° C 1 min. (polymerization) 20 Cycles

One tenth of the above PCR reaction was fractionated on a 3% NuSieve Agarose gel. A DNA band of the predicted size (172 base pairs) was observed. This gel was then "Southern" blotted onto GeneScreen Plus nylon membrane under denaturing conditions and hybridized to ^{32}P end-labeled chain 1 specific oligonucleotide probe (Fel 1) in 6 X SSC at 35 degrees C, and washed in 2 X SSC at 48 degrees C. The 173 base pair band hybridized to the chain 1 specific probe.

The remainder of the PCR reaction was restriction digested with Cla I and Xho I and fractionated over a preparative 3% NuSieve agarose gel and the 173 base pair band excised. The fragment was ligated into Cla I/Xho I digested Bluescript plasmid (Stratagene), and subjected to Sanger/dideoxy DNA sequence analysis using a Sequenase kit (US Biochemicals). The data from this analysis shown in Figure 1 demonstrated that the sequence of the PCR amplified DNA fragment, when translated, is in agreement with an internal portion of the protein sequence of chain 1 of TRFP.

The 3' end of the chain 1 cDNA encoding TRFP was cloned according to the RACE PCR method. Frohman, M.A., Dush, M.K., and Martin, G.R. Science 85: 8998-9002. (1988).

First strand cDNA synthesis was performed with 1 μg

of poly A plus RNA isolated from a pooled sample of cat parotid and mandibular glands using the EDT primer with Superscript reverse transcriptase.

RACE PCR amplification of the carboxy terminal portion of chain 1 was carried out using primer 3 and the ED primer as the 5' and 3' specific primers respectively. Primers were used with a Perkin Elmer/Cetus PCR kit to amplify an aliquot of the above cDNA using the following cycle:

94° C 1 min. (Denaturation)
55° C 1 min. 30 sec. (Annealing)
72° C 1 min. (polymerization) 30 Cycles

One tenth of the above PCR reaction was fractionated on a 2% agarose gel. After "Southern" blotting of the gel onto GeneScreen Plus nylon membrane and hybridization to a chain 1 specific oligonucleotide probe (Fel 1), as above, no bands that could be candidates for cDNAs encoding the 3' portion of the TRFP Chain 1 were detected.

A second PCR reaction with cycling identical to that used for the first amplification was performed with a 1/100th aliquot of the initial PCR reaction products as template and primer 4 (encoding amino acids just 3' of those encoded in primer 3) and the ED oligonucleotide as primers. This "nested" PCR rection served to specifically reamplify products from the primary PCR reaction derived from TRFP chain 1 cDNA.

One tenth of this second PCR reaction was fractionated on a 2% agarose gel. After "Southern" blotting of the gel onto GeneScreen Plus nylon and hybridization to a chain 1 specific oligonucleotide probe, as above, a DNA band about 350 base pairs in length was detected.

The remainder of the second PCR reaction was

restriction digested with Cla I and Xba I, and fractionated over a preparative 1% SeaPlaque agarose gel and the 350 base pair band excised. The fragment was ligated into Cla I/Xba I digested Bluescript plasmid (Stratagene), and subjected to Sanger/dideoxy DNA sequence analysis using a Sequenase kit (US Biochemicals). The data from this analysis shown in Figure 1 demonstrates the sequence of PCR amplified 350 base pair DNA fragment, when translated, is in agreement with the protein sequence at the carboxy terminus of chain 1 of TRFP. The DNA sequence analysis also reveals a stop codon adjacent to the cysteine codon at position 72, indicating the protein sequence analysis of chain 1 of TRFP had been done in its entirety. In addition, 3' untranslated DNA sequence of the 350 base pair fragment contains a prototypical polyadenylation signal characteristic of the 3' end of a cDNA.

Primers and Probes

primer 1

5'- TATCGATGAAATTTG$^{T}_{C}$CC$^{A}_{T}$GC$^{A}_{T}$GT- 3'
(Cla I)

primer 2

5'- GCTCGAG$^{G}_{A}$TC$^{T}_{C}$TC$^{T}_{C}$TC$^{A}_{T}$GTCAT- 3'
(Xho I)

primer 3

5'- GGAATTCATCGATGTGAAGAGGGATCTATTC- 3'
(EcoR I, Cla I)

primer 4

5'- GGATCGATGAATTCTATTCCTGACGGGAACCC- 3'
(Cla I, EcoR I)

EDT primer

5'- GGAATTCTCTAGACTGCAGGT$_{15}$- 3'
(EcoR I, Xba I, Pst I)

ED primer

5'- GGAATTCTCTAGACTGCAGGT- 3'
(EcoR I, Xba I, Pst I)

Fel 1 probe

5'- GA$^{T}_{C}$GA$^{G}_{A}$TA$^{T}_{C}$GT$^{G}_{A}_{T}_{C}$GA$^{G}_{A}$CA$^{G}_{A}$GT- 3'

Cloning of TRFP chain 2 cDNAs

First strand cDNA was synthesized with a commercial kit using oligo (dT) priming of mRNA prepared from a pool of the parotid/mandibular glands of five cats. An internal sequence of chain 2 was determined using MOPAC.

Two redundant oligomers were synthesized based on protein sequence of human T cell reactive feline protein Chain 2:

Oligomer #56. 5' CGGAATTCAA$^{G}_{A}$ATGGCIGA$^{G}_{A}$AC$^{G}_{A}_{T}_{C}$TGY$^{T}_{C}$C,

which coresponded to coding strand sequence encoding amino acids 2-8 (KMAETCP) and

Oligomer #57. 5' CGGGCTGCAGTA$^{G}_{A}$CA$^{G}_{A}$TC$^{T}_{C}$TGIAT$^{T}_{C}$TT$^{T}_{C}$TTCAT,

which corresponded to non-coding strand sequence complimentary to amino acids 42-48 (MKKIQDCY)

Oligomer 56 had an Eco RI restriction site added (underlined) and oligomer 57 had a Pst I restriction site added (underlined) for cloning purposes. Inosine (I) was used once in each oligomer to reduce the redundancy of the final oligomers as described in Knoth et al. 1988. Nucl. Acids Res. 16:10932.

PCR was performed using 100 pmol of each primer plus first strand cDNA using the following conditions for amplification:

Denature at 94 degrees C for 1 min.; primer anneal at 45 degrees C for 1.5 min.; elongate at 72 degrees C for 2 min.; repeat cycle 4 tim s.

Denature as above; anneal at 55 degrees C for 1.5 min.; elongate as above; repeat second cycle 19 times (total of 25 cycles).

Two cDNA clones containing Chain 2 sequence were identified. Both clones had identical sequence. The prototype clone is F2.m.

The COOH end of the chain 2 cDNA encoding TRFP was cloned according to the RACE PCR method. First strand cDNA was synthesized from mRNA as described above.

Oligomers used in amplification of Chain 2 were:

Oligomer #59. 5' GGATCGATGAATTCGGTGGCCAATGGAAATG, which corresponded to coding strand sequence encoding amino acids 19-23 (VANGN) of Chain 2 and contained Cla I and Eco RI restriction sites for cloning purposes.

Oligomer #61. 5' ATTACTGTTGGACTTGTCCCT, which corresponded to amino acids 23-28 (LLLDLS) of Chain 2, and ED/EDT primers described above.

Two PCR reactions were carried out using "nested primers." The primary PCR reaction used 100 pmol of oligomer 59 and 100 pmol of the ED and EDT primers in a 3:1 ratio. Amplification conditions were the same as those used in obtaining internal Chain 2 sequence. 0.01 volume of the primer PCR was reamplified using 100 pmol of oligo #61 (a "nested" primer) and 100 pmol of the ED primer using the standard conditions.

The amplified fragment was cloned and sequenced to give the COOH end of Chain 2. There are two prototype clones: F15.a and F15.d. F15.a matched one protein sequence of the dominant protein sequence for Chain 2 while F15.d matched a second protein sequence. F15.a has been called the "Long" sequence and F15.d has been called the "Short" sequence. There are 7 clustered amino acid differences between F15.a and F15.d including 5 amino acid changes and two amino acid deletions in F15.d

relative to F15.a (see Figures 6 and 7).

Chain 2 has been isolated from the mRNA from two cat skins as well as from mRNA from pooled salivary glands. The skin samples were sampled separately. Skin A (one of the five cats used in making the salivary gland pool) had mRNA encoding only the short form of Chain 2. Skin B (from a sixth cat not part of the five used in making the salivary gland pool) contained mRNA for both the Short and Long forms of Chain 2 in a 3:1 (S:L) ratio. A third form of Chain 2 has been found in the skin. This is called "ST" for Short Truncated (Figure 5). ST has 16 contiguous amino acid differences from the short form and has deleted the last 10 amino acids of the Short sequence. Examples of this clone have been found in mRNA from both Skin A and Skin B. Chain 2 Long is the dominant form of Chain 2 in the salivary glands (23/24 clones). Chain 2 Short is the dominant form of Chain 2 in the skin (20/25 clones from two cats), while the Long form (0/13 clones in Skin A and 3/12 clones in skin B) and the ST form (2/25 clones from two cats) appear to be minor forms. A summation of the complete nucleotide sequence of TRFP chains 1 and 2 derived by the methods cited above is presented in Figures 1-5.

Polymorphism in the long form of chain 2 was detected in the skin mRNA from one cat. This polymorphism involved the substitution of a Leucine for Isoleucine at amino acid 55 and a Threonine for a Methionine at position 74.

Cloning the NH_2 terminals of TRFP chain 1 and chain 2

First and second strand cDNA was synthesized with a commercial kit using oligo dT priming of mRNA prepared from a pool of the parotid/mandibular glands of five cats. The double-stranded cDNA was blunted and then

blunt-end ligated to annealed oligomers #68 and #69 (see below). These oligomers, described in Rafnar et. al., J. Biol. Chem., in press, were designed to utilize the "Anchored PCR" as described by Frohman et. al. 1988. Proc. Natl. Acad. Sci. USA 85:8998-9002 and modified by Roux and Dhanarajan. 1990 BioTech. 8:48-57. These oligonucleotides are not entirely homologous and, thus, will not self-prime. The oligomers will blunt end to every cDNA.

Oligomer #68. Template, Blunt Anchor
5' GGGTCTAGAGGTACCGTCCGATCGATCATT

Oligomer #69. Linker, Blunt Anchor
5' p-AATGATCGATGCT

Oligomer #64 Anchor Primer (AP) was
5' GGGTCTAGAGGTACCGTCCG

Cloning the NH_2 terminal of TRFP Chain 2

Two oligomers based on internal chain 2 nucleotide sequence were synthesized:
Oligomer #60. 5' CGGGCTCGAGCTGCAGCTGTTCTCTCTGGTTCAGT, which corresponded to non-coding strand sequence complementary to that encoding amino acids 35-40 (TEPERT), and
Oligomer #70. 5' GGGCTGCAGATTCTAGTCAGCCTGATTGA, which corresponded to non-coding strand sequence of the 3' UT region. Both oligomers matched the antisense strand sequence and contained Pst I and Xho I restriction sites (underlined, Oligomer 60) or Pst I (underlined, Oligomer 70) for cloning purposes.

Two PCR reactions were carried out using "nested primers". Amplification conditions were the same as those used for MOPAC. The 1° amplication reaction was

done with oligomers #64 (AP) and #70. 0.01 vol of the 1° PCR product was reamplified with oligomers #64 and #60 (which is "nested," i.e. internal, relative to oligo #70). Amplified material was recovered, cloned and sequenced.

Cloning the NH_2 terminal of TRFP chain 1

One oligomer based on internal chain 1 sequence was synthesized:
Oligomer #66, 5' GGGCTCGAGCTGCAGTTCTTCAGTATTCTGGCA, corresponded to non-coding strand sequence complementary to that encoding amino acids 38-43 (ARILKN) of chain 1. The restriction sites Pst I and Xho I were added for cloning purposes.

Two PCR reactions were carried out using "nested primers". Amplification conditions were the same as those used for MOPAC. 1° PCR was performed with primer 2 (described above) and #64 (AP). 0.01 vol of the 1° PCR product was reamplified with oligomers #64 and #66 (which is "nested," i.e. internal, relative to primer 2). Amplified material was recovered from the 2° PCR, cloned and sequenced. Two different 5' sequences were obtained and designated Leaders A and B (Figures 1 and 2).

Chain 1 with Leader A is a dominant sequence in both salivary gland and skin mRNA. It was not possible to detect chain 1 with Leader B sequence in the mRNA preparation from Skin A. Chain 1 with Leader B sequence was a minor component of the mRNA in both the pooled salivary gland and Skin B preparations.

EXAMPLE 3. Screening of a Cat Genomic DNA Library to Identify Clones Containing DNA Encoding the TRFP

An EMBL4 cat genomic library, using cat liver DNA as

starting material, was constructed using recommended procedures described in Frischauf, A.-M. et al. J. Mol. Biol. 170:827-842 (1983). The EMBL4 cat genomic library was screened using ^{32}P-radiolabelled chain 1 and chain 2 cDNA as probes. The library was plated out and screened, yielding individual genomic clones that hybridized to either chain 1 or chain 2 cDNA probes, but not to both. This hybridization pattern verified that the chain 1 and chain 2 cDNAs are products of different genes. Northern blot analysis of the cat salivary gland RNA probed with ^{32}P-radiolabelled TRFP chain 1 or 2 cDNA also demonstated the presence of the two separate mRNAs. The DNA sequence of the genomic clones (designated CTGch1 and CTGch2) was determined and confirmed the hybridization results.

Individual full length PCR generated chain 1 clones (Example 2) were shown to have two different sequences at their 5' end (see Figures 1 and 2). One interpretation is that chain 1 has two alternative leader sequences. The DNA sequence of the chain 1 genomic clone (CTGch1) has confirmed this interpretation and demonstrated that the single chain 1 gene possesses both alternative sequences closely-linked at the 5' end of the structural gene.

The DNA sequence of the chain 2 genomic clone (CTGch2) demonstrated the presence in the cat genome of different gene segments encoding the long and short forms of chain 2 (see Figures 3 and 4). The isolation of two genes encoding the TRFP chain 2 is consistent with the tissue specific expression of the two different mRNA forms in cat skin and salivary gland (Figures 3, 4 and 7; see Example 2).

Of note is that a comparison of the genomic sequences to that of isolated cDNAs demonstrated that the TRFP has sequence microheterogeneity.

EXAMPLE 4. Expression of Recombinant TRFP chains 1 and 2

cDNA clones encoding all or parts of TRFP chain 1 or chain 2 have been subcloned into E.coli expression vectors, specifically pSEM-1, -2 and -3 (Knapp, S. Broker, M. and E. Amann. BioTechniques 8: 280-281. (1990). These vectors carry a truncated form of the E. coli lac Z gene (lacZ'), encoding the N-terminal 375 amino acids of Beta-galactosidase (Beta-gal). cDNA clones encoding chain 1 and chain 2 of TRFP were altered using PCR methods such that the 5' end possessed an in-frame poly-histidine sequence followed by an asp-pro acid-sensitive bond. Cultures containing the chain 1 or 2 expression constructs produce substantial quantities of recombinant fusion protein products upon IPTG induction. The presence of the poly-histidine reporter group has allowed the recombinants to be highly purified using immobilized metal-ion affinity chromatography (Hochuli, E. et al. BioTechnology 6: 1321-1325 (1988). Mild-acid cleavage of the Asp-Pro site leads to the release of intact full-length TRFP chain 1 or chain 2 protein. Standard protein purification methods lead to substantial quantities of recombinant protein free of irrelevant sequences. Protein sequence analysis of the purified peptide have verified the authenticity of the sequence. Rabbit anti-peptide antisera directed against either chain 1 sequence (Fel 1, EITPAVKRDVDLFLTGT; Fel 2, DVDLFLTGTPDEYVEQV; Fel 4, NARILKNCVDAKMTEEDKE), or chain 2 sequences (Fel 18, LLLDLSLTKVNATEPERTAMKKIQDC), have been generated. The anti-peptide antisera react with the recombinant proteins (described above) on Western blots.

Recombinant chain 1 and chain 2 peptides, and fragments or modifications thereof, can be used as desensitizing therapeutants.

EXAMPLE 5. T cell Studies with Purified T Cell Reactive Protein

Peripheral blood mononuclear cells (PBMC) were purified from 60 ml of heparinized blood from cat allergic patients. PBMC were subsequently treated as described below, although in individual cases, the length of time of cultivation with IL-2 and/or IL-4 and the specific peptides used for stimulation varied.

10 ml of PBMC from patient 131 at 10^6/ml were cultured at 37°C for 7 days in the presence of 5 micrograms purified TRFP/ml RPMI-1640 supplemented with 5% pooled human AB serum. Viable cells were purified by Ficoll-Hypaque centrifugation and cultured for three weeks at 5 units recombinant human IL-2/ml and 5 units recombinant human IL-4/ml. The resting T cells were then restimulated (secondary) with 5 micrograms TRFP at 2 x 10^5/ml in the presence of irradiated (3500 Rads) autologous PBMC at a concentration of 5 x 10^5/ml for three days, purified by Ficoll-Hypaque centrifugation and grown in 5 units IL-2 and 5 units IL-4/ml for two weeks. For assay, 2 x 10^4 resting secondary T cells were restimulated (tertiary) in the presence of 5 x 10^4 irradiated (3500 Rads) autologous PBMC or 2 x 10^4 transformed B cells (20,000 Rads) with various concentrations of allergens or their fragments in a volume of 200 microliters in a 96-well round bottom assay plates for 3 days. Each well then received 1 microCurie tritiated (methyl) thymidine for 16 hours. The counts incorporated were collected onto glass fiber filters and processed for liquid scintillation counting.

Antigens used: T cell reactive feline protein (TRFP), Hollister-Stier cat epithelium skin test reagent (CST), IPC ragweed pollen extract (pollen), and synthetic peptides derived from the TRFP protein sequence (See

Figures 6 and 7). Significant T cell proliferation is generally regarded as greater than 2.5 times the media control (T cells and antigen presenting cells alone).

Alternatively, PBMC were treated as follows: the primary stimulated cells were cultured in IL-2/IL-4 for two weeks. The resting T cells derived from this culture were tested in a secondary assay with some, but not all, of the above allergens. The results of these assessments are shown in Tables 2 and 3. Figure 9 demonstrates that T cells from patient 131 respond to T cell epitopes present in the Fel 1, 5 and 8 peptides. This type of epitope analysis has allowed the definition of T cell epitopes present in TRFP. Using a larger panel of patients, we have demonstrated the dominant epitopes in a heterogeneous population by deriving a positivity index (PI). The PI is derived from the average stimulation index of the responding population multiplied by the percentage of individuals that demonstrate a positive response to that peptide. This analysis is shown in Table 2 and 3.

This data demonstrates that while most of the TRFP protein contains T cell epitopes capable of stimulating T cells from some individuals, there are major differences in the strength of the elicited T cell response obtained with different portions of the TRFP molecule. The data has shown that each epitope works in some individuals and that each individual has a characteristic response pattern. For example, it appears that Fel 28 is the weakest T cell epitope containing region of TRFP (Table 3), whereas the most dominant T-cell epitopes are contained by the Fel 8 peptide (Table 2).

Table 2

Response of Cat Allergic Patients to Chain #1 TRFP Peptides

Peptide	Amino acid	PI[1]	SI[2]	N[3]
Fel 1[4]	1 - 17, 3T	392	5.6	121
1-2	1 - 17	311	7.4	83
1-3	4 - 17	422	6.2	26
1-4	6 - 17	816	9.6	32
1-5	8 - 17	286	5.2	25
1-6	10 - 17	312	5.2	26
Fel 2	9 - 25	416	7.3	30
Fel 3[6]	18 - 33, 31P,32D	674	9.5	123
3-1	18 - 33	638	9.4	40
3-10	18 - 31	504	6.9	28
3-11	18 - 30	863	10.4	12
3-15	18 - 29	1040	10.4	13
3-13	18 - 28	690	11.5	14
3-14	18 - 27	260	6.2	10
Fel 8	1 - 30	1393	18.1	86
8-1	5 - 33	1374	15.1	47
8-2	6 - 33	1353	15.2	47
8-3	7 - 33	1437	16.9	47
Fel 14	18 - 43	1054	13.7	125
14-1	23 - 36	871	9.9	88
14-3	25 - 36	621	6.9	90
14-4	26 - 36	474	6.0	79
14-5	27 - 36	286	5.4	53
14-2	29 - 42	336	5.6	60

Table 2 continued

Fel 4	37 - 55	601	7.7	120
4-1	37 - 52	822	9.9	20
4-2	37 - 49	378	6.2	15
4-3	37 - 46	185	3.7	13
Fel 30-1	25 - 49	268	5.6	23
30-2	25 - 48	248	4.2	22
30-3	25 - 47	230	4.8	23
30-4	29 - 55	1079	11.6	44
30-5	29 - 54	792	11.0	43
30-6	29 - 53	415	6.2	43
30-7	26 - 55	339	5.3	14
30-8	28 - 55	262	4.1	14
Fel 15	44 - 60	440	11.0	60
Fel 23	51 - 66	343	6.6	63
Fel 21[5]	56 - 70, 70R	360	5.8	66

[1]PI: Average SI of all responding patients tested multiplied by the percent of those patients with a positive response

[2]SI: Average of the cpm of T cell and antigen presenting cell proliferation to the antigen divided by cpm of T cells and antigen presenting cells alone from responding patients. An SI of ≥ 2.5 is considered positive.

[3]N: Number of patients tested.

[4]: Amino acid 3 changed to T.

[5]: Amino acid 70 changed to R.

6: Amino acids 31 and 32 changed to P and D, respectively.

Table 3

Response of Cat Allergic Patients to Chain #2 TRFP peptide

Peptide	Amino acid	PI	SI	N
Fel 16	1 - 22	283	5.9	52
Fel 17	12 - 33	421	6.1	114
Fel 32-1	12 - 24	442	6.6	21
32-2	14 - 24	424	5.3	20
32-3	16 - 24	270	3.7	22
Fel 18	23 - 48	466	6.3	99
Fel 33-1	26 - 36	340	5.4	63
33-2	26 - 38	210	4.2	50
33-3	26 - 40	235	5.0	47
Fel 31-1	14 - 40	733	9.4	36
31-2	14 - 39	599	8.1	35
31-3	14 - 38	598	8.3	36
31-4	14 - 37	622	8.4	35
31-5	14 - 36	539	7.6	37
31-6	15 - 40	295	4.4	33
31-7	15 - 36	267	5.8	33
Fel 20-1	34 - 59	395	5.9	79
Fel 25	49 - 68	350	7.6	56
Fel 28	60 - 82	94	3.6	43

Table 3 continued

Fel 28-1[1]	60 - 82	176	5.5	44
Fel 29	74 - 92	259	5.5	47

[1] Based on short form chain 2 sequence (C2S)

EXAMPLE 6. Induction of T cell anergy by a TRFP T cell epitope containing peptide

Exposure of peptide specific T cells to their specific peptide can induce anergy to the protein containing the T cell epitopes (Jenkins, M.K., and Schwartz, R.H. J. Exp. Med 165:302-319 (1987)). It is predicted that any strong T cell epitope can be used to induce tolerance to the whole allergen. This would result in the inability of the individual to respond to a natural allergen exposure. The individual would not respond by the stimulation of helper T cells. The lack of helper T cells would result in an altered lymphokine response and/or the absence of an IgE response and, consequently, a reduced allergic response to cat allergens.

Patient 155 TRFP secondary primed T cells (2.5×10^6) were rested and cultured with (2.5×10^6) irradiated autologous Epstein Barr Virus transformed B cells (EBV) in 1 ml of complete RPMI with 10% AB serum in 12x75mm polypropylene snap cap tubes and increasing amounts of antigen over 5 consecutive days. The T cell cultures were exposed to 5μg/ml TRFP on day 0 and 5μg/ml, 10μg/ml, 10μg/ml and 20μg/ml thereafter. The peptide treated cultures were exposed to 1μg/ml peptide on day 0 and 1μg/ml, 1μg/ml, 2μg/ml and 5μg/ml thereafter. In addition, 0.5ml of fresh media was replaced on day 2. The cells were then washed and set up for a proliferation

assay with $2x10^4$ T cells and $2x10^4$ irradiated (2500 Rad) autologous EBV and various doses of antigen. T cell proliferation was measured as incorporation of tritiated thymidine at day 9. The induction of in vitro anergy or tolerance is demonstrated in Table 4. This experiment demonstrates the ability of TRFP and peptides thereof to induce anergy or tolerance in antigen specific T cell lines.

Table 4

Antigen Response of a TRFP-primed T cell culture exposed to a Fel 8-3 or TRFP.

	T cell proliferation (cpm) following Antigenic challenge:			
Tolerance treatment:	-	TRFP	Fel 8-3	Ragweed Peptide
-	1,530	58,890	58,150	2,130
Fel 8-3	1,270	12,320	3,850	5,130
TRFP	2,190	33,160	36,030	6,670
Ragweed Pep.	920	64,050	45,020	3,590

EXAMPLE 7. Cytokine profiles of T cells responsive to purified TRFP or synthetic peptides derived from the TRFP protein sequence

Peripheral blood mononuclear cells (PBMC) were purified from cat-allergic patients as described in Example 5. Five x 10^6 PBMC were cultured at 2 x 10^6/ml for 36 hours in the presence of medium only, 20 micrograms purified TRFP/ml or 50 micrograms peptide/ml. The cells were then washed two times with phosphate buffered saline (PBS, pH 7.2) and lysed with 2 ml 0.4 M

guanidinium isothiocyanate, 0.5% Sarkosyl, 5 mM sodium citrate, 0.1 M 2-mercaptoethanol, pH 7.0. The lysate was then forced through a 26 gauge needle to shear genomic DNA, and was layered onto a 2 ml cushion of 5.7 M CsCl, 0.01 M EDTA, pH 7.5 in diethylpyrocarbonate (DEPC)-treated water. The lysate was centrifuged at 35,000 RPM in a Beckman SW41 rotor for 18 hours at 20°C. The RNA pellet was resuspended in 0.4 ml TE (10 mM Tris, 1 mM EDTA) pH 7.5, 0.1% SDS, and then extracted three times with 0.5 ml phenol/0.2 ml chloroform. The RNA was then precipitated on dry ice with 2.5 volumes ice-cold ethanol, 1/10 volume 3 M sodium acetate, pH 5.2 in DEPC-treated water, rinsed once with 70% ethanol in DEPC-treated water, and dried. The RNA pellet was resuspended in 2 microliters TE, pH 7.5 in DEPC-treated water.

Total cellular RNA was converted to cDNA using the Superscript kit (BRL, Bethesda, MD). Two microliters of RNA were added to one microliter oligo $(dT)_{12-18}$ (500 micrograms/ml) and 9 microliters water. The sample was heated to 70°C for 10 minutes and ice quenched. Four microliters 5X buffer were added to the sample along with 2 microliters 0.1 M DTT and 1 microliter dNTP mix (10 mM each, dATP, dCTP, dGTP, dTTP). One microliter reverse transcriptase (200U) was added and the reaction was carried out for one hour at 42°C. The reaction was terminated by incubation of the sample at 90°C for five minutes and stored at -80°C.

Ten-fold serial dilutions of T cell cDNA in 10 mM TRIS, pH 7.5 were amplified using the standard kit and protocol recommended by Perkin Elmer-Cetus (Redwood City, CA). Each sample received 26.65 microliters water, 5 microliters 10X PCR buffer, 8 microliters of dNTP mix (1.25 mM each of dATP, dCTP, dGTP and dTTP), 0.1 microliter alpha ^{32}P-dCTP (3000 Ci/mmol), 0.25 microliter

AmpliTaq, 5 microliters cDNA and 5 microliters cytokine-specific 5' and 3' primers (20 micromolar). The primers used for most cytokines and the beta-actin control were purchased from Clontech (Redwood City, CA). The human IL-4 primers were purchased from Research Genetics (Huntsville, AL) and had the following sequences:

5' hIL-4 primer

5'-GTC-CAC-GGA-CAC-AAG-TGC-GAT-ATC-ACC-3'

3' hIL-4 primer

5'-GTT-GGC-TTC-CTT-CAC-AGG-ACA-GGA-ATT-C-3'

The reactions were carried out after overlaying each sample with one drop of mineral oil, with the following program in a programmable thermal cycler (MJ Research, Cambridge, MA).

Step	Temperature	Time
1	94°C	1 min
2	60°C	1 min
3	72°C	2 min
4	cycle to step 1 29 times	
5	72°C	7 min
6	4°C	hold

The PCR products were extracted once with 25 microliters chloroform and 25 microliters of each sample were then electrophoresed on an 8% polyacrylamide gel at 250 V. The gel was dried and exposed to pre-flashed x-ray film. Several exposures of each gel were then scanned using a Shimadzu flying spot laser densitometer. Values on the linear portion of the titrations were then compared to the medium control values to obtain a stimulation index for each sample. Primers for beta-actin were included as a control for general cDNA levels in each sample. Where the medium control values are not detectable, the lowest measurable response was set at 1.00. In other experiments, levels of cytokine cDNA can

be compared directly with previously amplified cytokine-specific cDNAs as standards. Thus, absolute levels of particular cytokine cDNAs can be compared from one sample to another and from one experiment to another. Results of a representative experiment are shown in Table 5. In this experiment, IL-2, IL-4 and IFN-gamma levels were measured. As shown, in this particular cat-allergic patient, peptides such as Fel 18 generate more IL-4 than certain other TRFP-derived peptides (Fel 14 and Fel 17). This analysis will be expanded to studies of other cytokines involved in the generation of allergic responses, such as IL-5, IL-8, IL-9, TGF-beta. Samples of cDNA from each treatment can also be saved for later analysis once additional cytokines are identified and sequenced. Peptides generating a spectrum of cytokines favorable for the generation of allergic responses can be avoided for therapeutic use in the treatment of cat allergy. Similarly, TRFP-derived peptides that are shown to generate cytokines which dampen the allergic response, such as IFN-gamma and IL-10, can be selected for treatment of cat allergy.

Table 5

IL-2, IFN-γ and IL-4 Measurements by PCR

Patient 409 primary 36 hour culture

	STIMULATION INDEX			RATIO	
Treatment	IL-2	IFN-γ	IL-4	IL-2/IL-4	IFN/IL-4
Medium	--	1.0	1.0		
TRFP	--	2.1	3.8		0.9
Fel 8	1.0	1.8	0.3	3.3	6.0
Fel 14	106.9	75.0	1.9	56.3	39.5
Fel 4	--	25.8	10.0		2.6
Fel 21	23.9	41.6	3.2	7.5	13.0
Fel 17	315.1	82.4	7.5	42.0	11.0
Fel 18	4.6	5.6	12.0	0.4	0.5
Fel 20-1	--	12.0	4.6		2.6
Fel 25	2.9	12.7	2.5	1.2	5.1
Fel 28	--	3.8	0.5		7.6
Fel 29	1.2	3.0	1.1	1.1	2.7

CLAIMS

1. Substantially pure covalently linked human T cell reactive feline protein of approximately 40,000 MW, or a portion thereof.

2. Human T cell reactive feline protein having all or a portion of an amino acid sequence selected from the group consisting of:

a) C I M K G A R V L V L L W A A L L L I W G
G N C E I C P A V K R D V D L F L T G T P
D E Y V E Q V A Q Y K A L P V V L E N A R
I L K N C V D A K M T E E D K E N A L S L
L D K I Y T S P L C;

b) A W R C S W K R M L D A A L P P C P T V A
A T A D C E I C P A V K R D V D L F L T G
T P D E Y V E Q V A Q Y K A L P V V L E N
A R I L K N C V D A K M T E E D K E N A L
S L L D K I Y T S P L C;

c) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L
L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M T
T I S S S K D C M G E A V Q N T V E D L K
L N T L G R;

d) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L
L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M I
A I N E Y C M G E A V Q N T V E D L K L N
T L G R; and

e) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L

L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M P
S T N I A W V K Q F R T P.

3. Human T cell reactive feline protein of Claim 2 which is modified human T cell reactive feline protein.

4. Human T cell reactive feline protein of Claim 1 capable of modifying, in a cat allergen-sensitive individual to whom it is administered, the allergic response to a cat allergen.

5. Human T cell reactive feline protein of Claim 4 capable of modifying B-cell response to a cat allergen, T-cell response to a cat allergen, or both.

6. An isolated allergenic peptide of a human T cell reactive feline protein comprising all or a portion of an amino acid sequence selected from the group consisting of:
   a) T cell reactive feline protein chain 1, as represented in Figure 6;
   b) T cell reactive feline protein chain 2 long, as represented in Figure 7;
   c) T cell reactive feline protein chain 2 short, as represented in Figure 7;
   d) T cell reactive feline protein chain 2 short truncated, as represented in Figure 7; and
   e) functional equivalents thereof.

7. An isolated peptide of Claim 6 which does not bind IgE.

8. An isolated peptide of Claim 6 which stimulates T cells from a cat allergic individual.

9. A modified peptide of a human T cell reactive feline protein which does not bind IgE.

10. A modified peptide of a human T cell reactive feline protein which stimulates T cells.

11. A modified peptide of a human T cell reactive feline protein which does not bind IgE and which stimulates T cells.

12. A modified peptide of Claim 9, 10 or 11 capable of desensitizing a cat allergen-sensitive individual to whom it is administered.

13. A modified peptide of a human T cell reactive feline protein which, when administered to a cat-sensitive individual, reduces the allergic response of the individual to cat allergen.

14. A modified peptide of a human T cell reactive feline protein comprising at least two T cell reactive epitopes joined by a linker.

15. The modified human T cell reactive feline protein of Claim 14 wherein at least one of the T cell reactive epitopes is a chain 1 epitope

and at least one of the T cell reactive epitopes is a chain 2 epitope.

16. Recombinant human T cell reactive feline protein chain 1, or a portion thereof.

17. Recombinant human T cell reactive feline protein chain 2, or a portion thereof.

18. Recombinant human T cell reactive feline protein of Claim 16 or 17 having:
    a) all or a portion of the amino acid sequence of Figure 6, or
    b) all or a portion of the amino acid sequence of Figure 7.

19. Recombinant human T cell reactive feline protein of Claim 16 or 17 additionally comprising, at the 5' end thereof, an in-frame polyhistidine sequence followed by an asp-pro acid-sensitive bond.

20. Recombinant human T cell reactive feline protein of Claim 16 or 17 expressed in E. coli as a recombinant fusion protein product.

21. Recombinant T cell reactive feline protein which comprises an amino acid sequence of human T cell reactive feline protein chain 1 linked to an amino acid sequence of human T cell reactive feline protein chain 2 and modifications of said amino acid sequences.

22. A human T cell reactive feline peptide having an amino acid sequence selected from the group consisting of:

a) amino acids of chain 1 of feline protein, selected from the group consisting of:

1) amino acids 1-17, wherein amino acid 3 is T;
2) amino acids 1-17;
3) amino acids 4-17;
4) amino acids 6-17;
5) amino acids 8-17;
6) amino acids 10-17;
7) amino acids 9-25;
8) amino acids 18-33, wherein amino acid 31 is P and amino acid 32 is D;
9) amino acids 18-33;
10) amino acids 18-31;
11) amino acids 18-30;
12) amino acids 18-29;
13) amino acids 18-28;
14) amino acids 18-27;
15) amino acids 1-30;
16) amino acids 5-33;
17) amino acids 6-33;
18) amino acids 7-33;
19) amino acids 18-43;
20) amino acids 23-36;
21) amino acids 25-36;
22) amino acids 26-36;
23) amino acids 27-36;
24) amino acids 29-42;
25) amino acids 37-55;
26) amino acids 37-52;

27) amino acids 37-49;
28) amino acids 37-46;
29) amino acids 25-49;
30) amino acids 25-48;
31) amino acids 25-47;
32) amino acids 29-55;
33) amino acids 29-54;
34) amino acids 29-53;
35) amino acids 26-55;
36) amino acids 28-55;
37) amino acids 44-60;
38) amino acids 51-66; and
39) 56-70, wherein amino acid 70 is R;

b) amino acids of chain 2 long of human T cell reactive feline protein, selected from the group consisting of:
1) amino acids 1-22;
2) amino acids 12-33;
3) amino acids 12-24;
4) amino acids 14-24;
5) amino acids 16-24;
6) amino acids 23-48;
7) amino acids 26-36;
8) amino acids 26-38;
9) amino acids 26-40;
10) amino acids 14-40;
11) amino acids 14-39;
12) amino acids 14-38;
13) amino acids 14-37;
14) amino acids 14-36;
15) amino acids 15-40;
16) amino acids 15-36;
17) amino acids 34-59;

18) amino acids 49-68;
19) amino acids 60-82;
20) amino acids 74-92;

c) amino acids 60-82 of chain 2 short of human T cell reactive feline protein; and

d) modifications of the amino acid sequences in a), b) or c) above.

23. A human T cell reactive feline peptide which has a stimulation index of at least approximately 4.0 or a positivity index of at least approximately 250.

24. A human T cell reactive feline peptide which is capable of producing anergy in a cat allergic individual to whom it is administered or is capable of modifying the lymphokine secretion profile of T cells in a cat allergic individual to whom it is administered.

25. Isolated DNA encoding human T cell reactive feline peptide having all or a portion of an amino acid sequence selected from the group consisting of:

a) C I M K G A R V L V L L W A A L L L I W G
G N C E I C P A V K R D V D L F L T G T P
D E Y V E Q V A Q Y K A L P V V L E N A R
I L K N C V D A K M T E E D K E N A L S L
L D K I Y T S P L C;

b) A W R C S W K R M L D A A L P P C P T V A
A T A D C E I C P A V K R D V D L F L T G
T P D E Y V E Q V A Q Y K A L P V V L E N
A R I L K N C V D A K M T E E D K E N A L

S L L D K I Y T S P L C;

c) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L
L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M T
T I S S S K D C M G E A V Q N T V E D K K
L N T L G R;

d) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L
L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M I
A I N E Y C M G E A V Q N T V E D L K L N
T L G R; and

e) D T M R G A L L V L A L L V T Q A L G V K
M A E T C P I F Y D V F F A V A N G N E L
L L D L S L T K V N A T E P E R T A M K K
I Q D C Y V E N G L I S R V L D G L V M P
S T N I A W V K Q F R T P.

26. A therapeutic composition comprising a human T cell reactive feline protein or peptide.

27. A therapeutic composition of Claim 26 wherein the human T cell reactive feline protein or peptide has an amino acid sequence selected from the group consisting of:

a) the amino acid sequences of Claim 2;
b) the amino acid sequences of Claim 22; and
c) modifications of the amino acid sequences of a) or b).

28. An antibody specifically reactive with an allergenic peptide of a human T cell reactive feline protein or peptide.

29. An antibody of Claim 28 which is specifically reactive with an allergenic peptide of a human T cell reactive feline protein or peptide having an amino acid sequence selected from the group consisting of:
a) the amino acid sequences of Claim 2; and
b) the amino acid sequences of Claim 22.

30. A peptide derived from human T cell reactive feline protein having all or a portion of the amino acid sequence of Figures 1 through 5, or an immunogenic modified peptide having all or a portion of the amino acid sequence of Figures 1 through 5, for use as a medicament for treating sensitivity to cat allergen.

31. A peptide according to Claim 30 wherein the peptide is a peptide of Claim 16 or Claim 17.

32. The peptide of Claim 13 for use as a diagnostic agent for determining the allergic response in the individual to said peptide.

33. An in vitro method of detecting in an individual sensitivity to a cat allergen, comprising combining a blood sample obtained from the individual with an isolated allergenic peptide of a feline T cell reactive protein, under conditions appropriate for binding of

blood components with the peptide, and determining the extent to which such binding occurs.

34. A method of Claim 33, wherein the extent to which binding occurs is determined by assessing T cell function, T cell proliferation, B cell function, binding of the peptide to antibodies present in the blood, or a combination thereof.

35. Use of a peptide derived from human T cell reactive feline protein having all or a portion of the amino acid sequence of Figures 1 through 5, for the manufacture of a medicament for treating sensitivity to cat allergen.

36. The use according to Claim 35 wherein the peptide is a peptide of Claim 16 or Claim 17.

37. Use of the peptide of Claim 13 for the manufacture of a diagnostic agent for determining the occurrence of an allergic response in the individual to said peptide.

FIGURE 1.
TRFP CHAIN 1, LEADER A

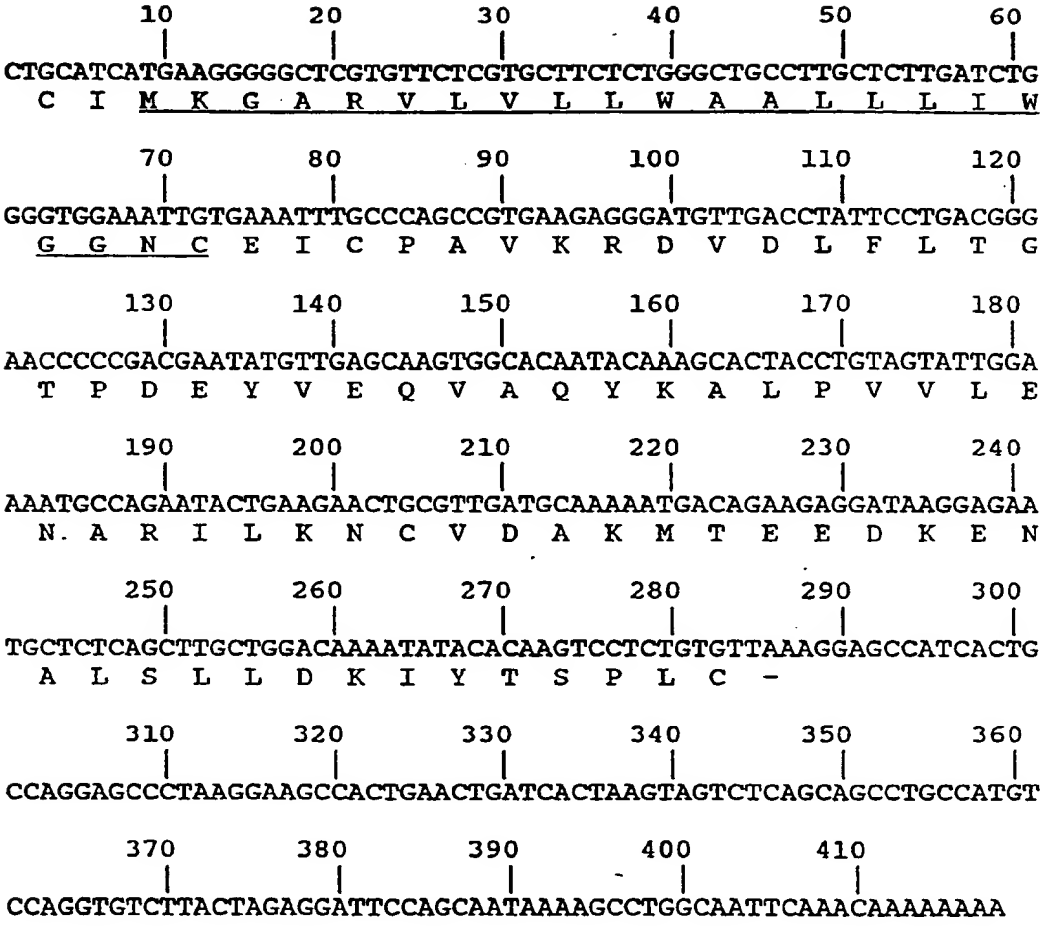

FIGURE 2.
TRFP CHAIN 1, LEADER B

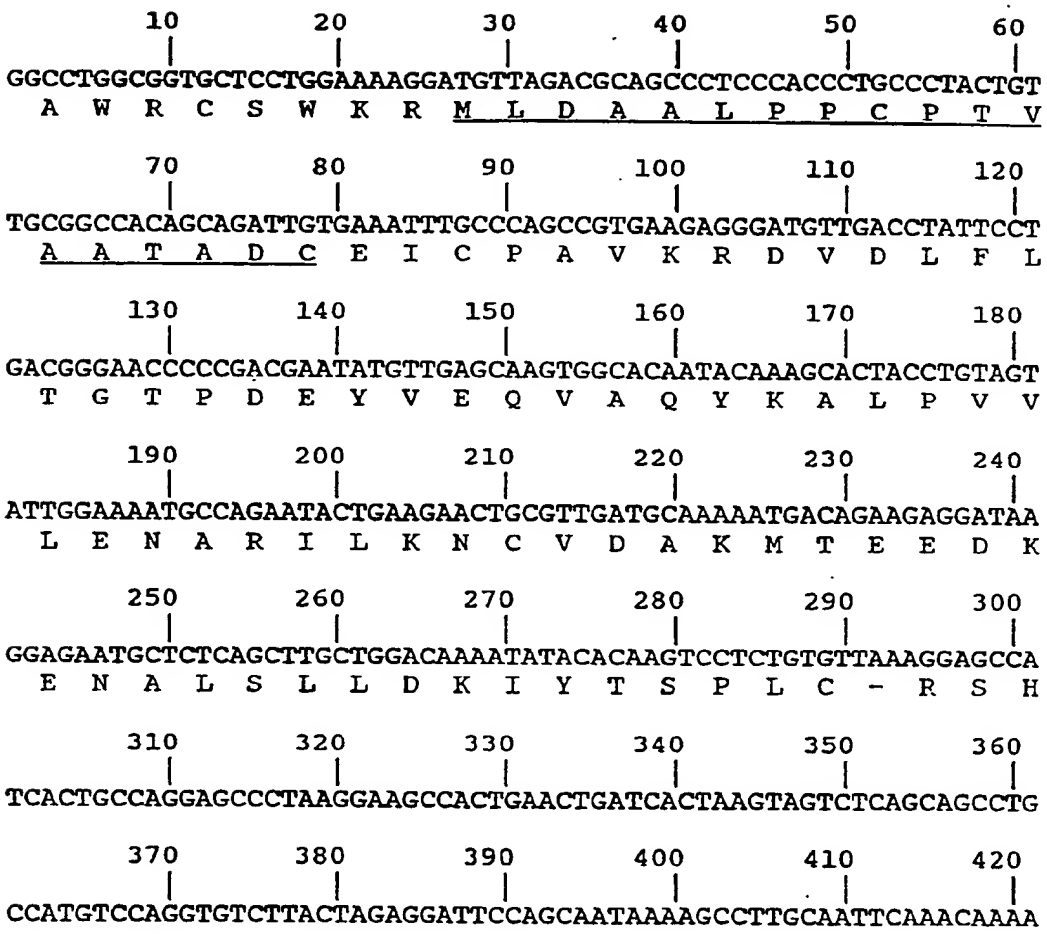

FIGURE 3.
TRFP CHAIN 2, LONG FORM

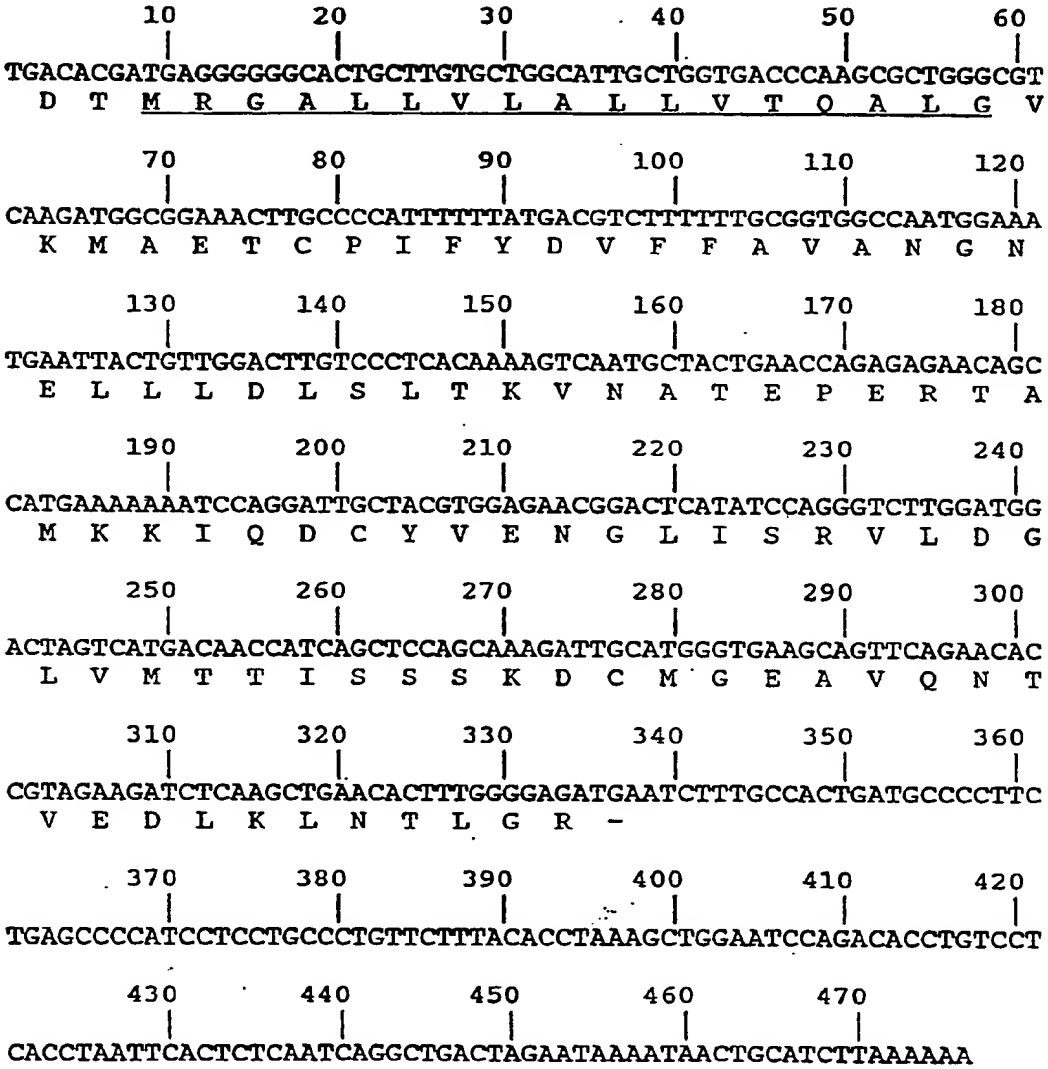

FIGURE 4.
TRFP I CHAIN 2, SHORT FORM

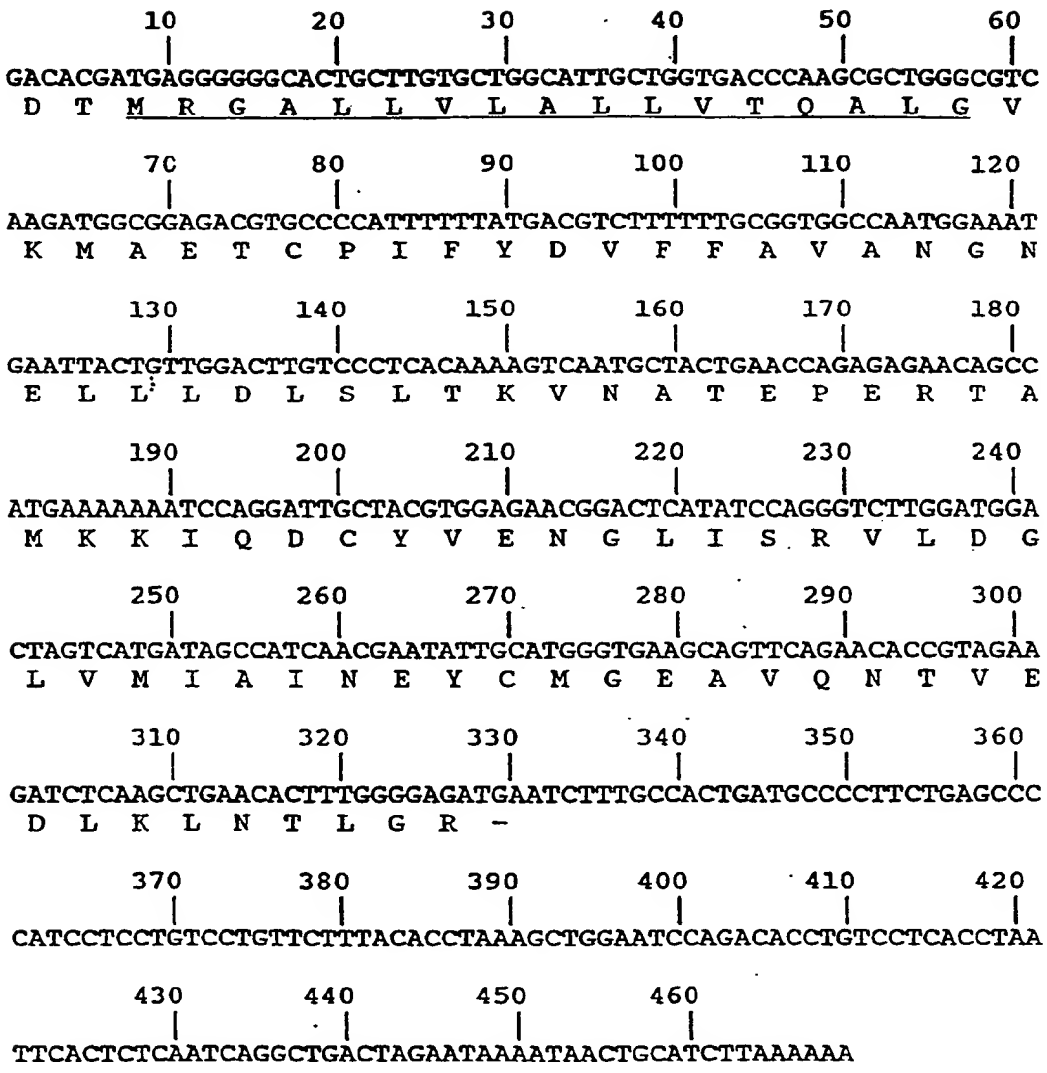

FIGURE 5.
TRFP CHAIN 2, TRUNCATED SHORT FORM

```
          10         20         30         40         50         60
          |          |          |          |          |          |
GACACGATGAGGGGGGCACTGCTTGTGCTGGCATTGCTGGTGACCCAAGCGCTGGGCGTC
 D  T  M  R  G  A  L  L  V  L  A  L  L  V  T  Q  A  L  G  V

          70         80         90         100        110        120
          |          |          |          |          |          |
AAGATGGCGGAGACGTGCCCCATTTTTTATGACGTCTTTTTTGCGGTGGCCAATGGAAAT
 K  M  A  E  T  C  P  I  F  Y  D  V  F  F  A  V  A  N  G  N

          130        140        150        160        170        180
          |          |          |          |          |          |
GAATTACTGTTGGACTTGTCCCTCACAAAAGTCAATGCTACTGAACCAGAGAGAACAGCC
 E  L  L  L  D  L  S  L  T  K  V  N  A  T  E  P  E  R  T  A

          190        200        210        220        230        240
          |          |          |          |          |          |
ATGAAAAAAATCCAGGATTGCTACGTGGAGAACGGACTCATATCCAGGGTCTTGGATGGA
 M  K  K  I  Q  D  C  Y  V  E  N  G  L  I  S  R  V  L  D  G

          250        260        270        280        290        300
          |          |          |          |          |          |
CTAGTCATGCCATCAACGAATATTGCATGGGTGAAGCAGTTCAGAACACCGTAGAAGATC
 L  V  M  P  S  T  N  I  A  W  V  K  Q  F  R  T  P  -

          310        320        330        340        350        360
          |          |          |          |          |          |
TCAAGCTGAACACTTTGGGGAGATGAATCTTTGCCACTGATGCCCCTTCTGAGCCCCATC

          370        380        390        400        410        420
          |          |          |          |          |          |
CTCCTGTCCTGTTCTTTACACCTAAAGCTGGAATCCAGACACCTGTCCTCACCTAATTCA

          430        440        450        460
          |          |          |          |
CTCTCAATCAGGCTGACTAGAATAAAATAACTGCATCTTAAAAAA
```

FIGURE 6.
TRFP CHAIN #1 PROTEIN SEQUENCE

```
                                -20                 -10
C1 Leader A          C I M K G A R V L V L L W A A L L I W G G N C

C1 Leader B          A W R C S W K R M L D A A L P P C P T B A A T A D C

                     5         10        15        20        25        30        35
C1  E I C P A V K R D V D L F L T G T P D E Y V E Q V A Q Y K A L P V V L
PRO. - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

                     40        45        50        55        60        65        70
C1  E N A R I L K N C V D A K M T E E D K E N A L S L L D K I Y T S P L C
PRO. - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
```

FIGURE 7.
TRFP CHAIN #2 PROTEIN SEQUENCES

```
                                                                      -10
C2 Leader                                             D T M R G A L L V L A L L V T Q A L G

                    5        10        15        20        25        30        35        40
C2L         V K M A E T C P I F Y D V F F A V A N G N E L L L D L S L T K V N A T E P E R T
C2S         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
C2ST                                                              - - - - - - - - - - - - -
PRO.        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - X - - - - - - -
```

FIGURE 7 (continued)

```
                45        50        55        60        65        70        75        80
C2L   AMKKIQDCYVENGLISRVLDGLVMTTISSSKDCMGEAVQN
C2S   ----------------------------IA-NE**Y-------
C2ST  ----------------------------PSTNIAWVKQFRTP
PRO.  ----------------------------TT--SS(  K)----
                                   IA--NE(
                85        90
C2L   TVEDLKLNTLGR
C2S   ------------
C2ST  TV
PRO.  AM
```

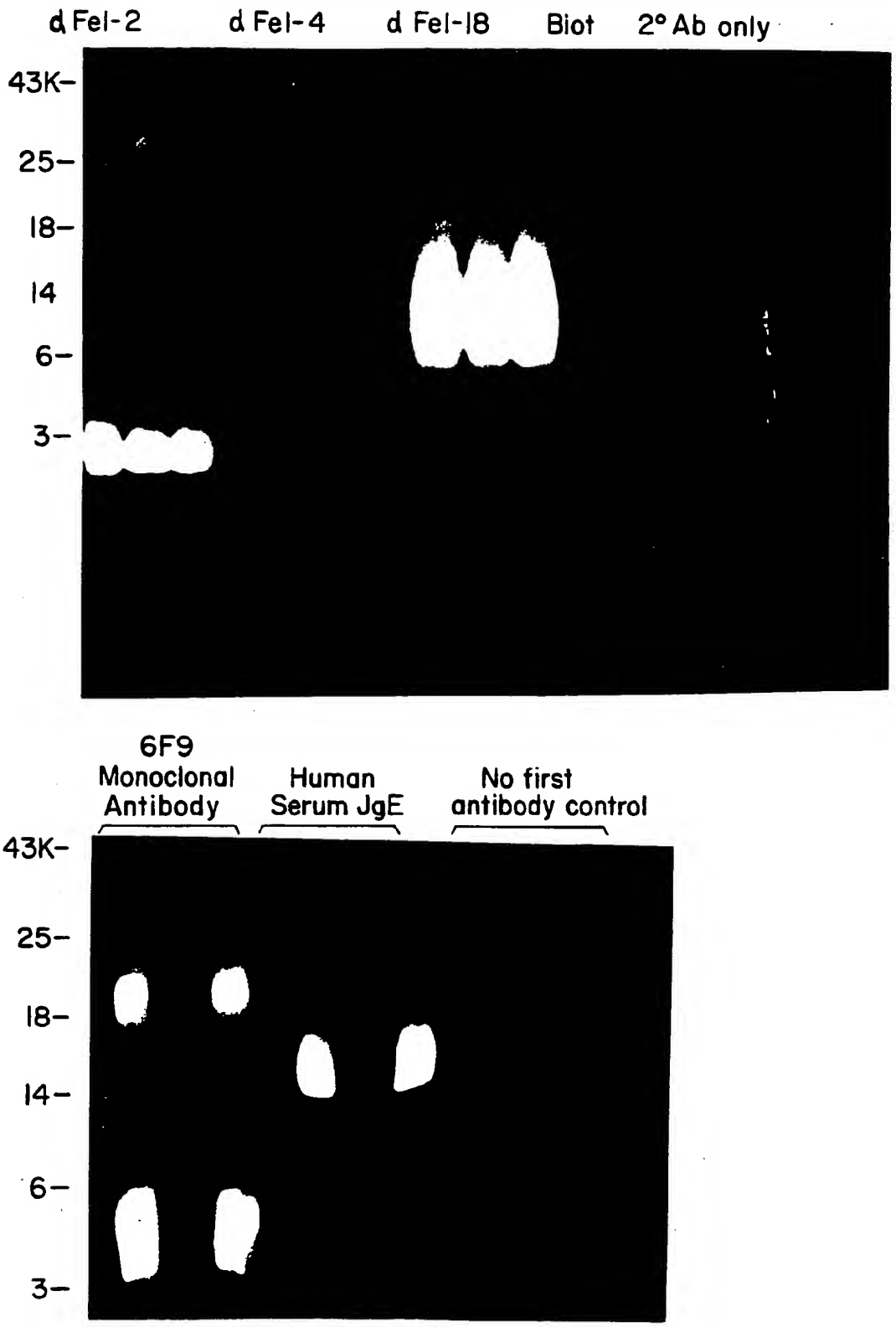

FIG. 8

SUBSTITUTE SHEET

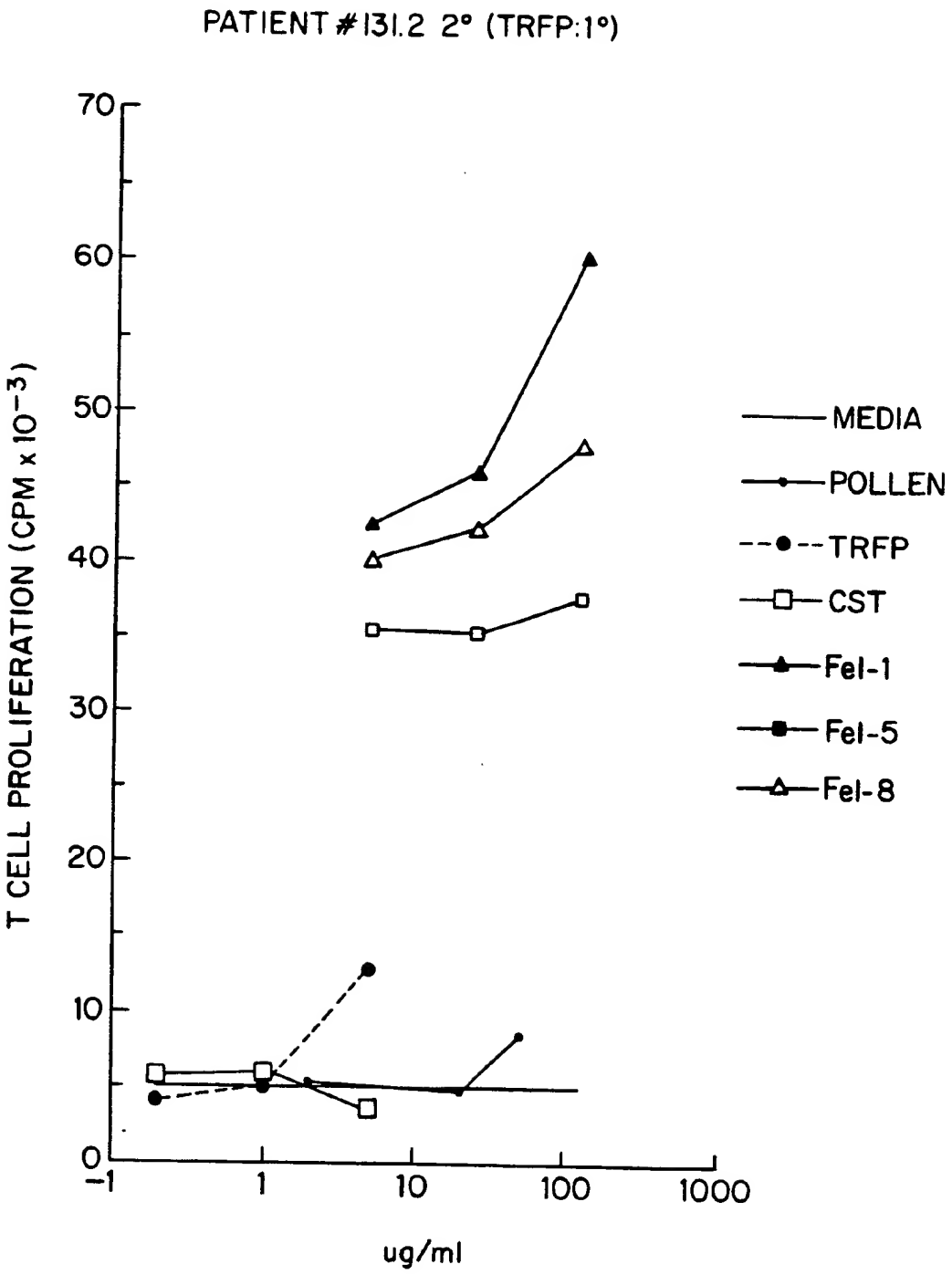
PATIENT #131.2 2° (TRFP:1°)
T CELL PROLIFERATION (CPM x 10⁻³)
70
60
50
40
30
20
10
0
-1
1
10
100
1000
ug/ml
MEDIA
POLLEN
TRFP
CST
Fel-1
Fel-5
Fel-8

Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/06548

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/06, A 61 K 39/35

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Dialog Information Services, File 155: Medline, NLM accession number 88116696, Chapman M.D. et al: "Monoclonal antibodies to the major *feline* *allergen* Fel d I. II. Single step affinity purification of Fel d I, N-terminal sequence analysis, and development of a sensitive two-site immunoassay to assess Fel d I exposure", & J Immunol (UNITED STATES) Feb 1 1988, 140 (3) p812-818.	1,4,5,9-11,16,23,24,28
Y		12,13,26,32,33,37
X	Dialog Information Services, File 155: Medline, NLM accession number 84110579, Didierlaurent A. et al: "Comparative study on *cat* *allergens* from fur and saliva", & Int Arch Allergy Appl Immunol 1984, 73 (1) p27-31.	1,4,5,9-11,16,23,24,28
Y		12,13,26,32,33,37

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th February 1991	22 FEB 1991
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: MISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4163778 (JOHN L. OHMAN ET AL.) 7 August 1979, see the whole document -- --------	12,13, 26,32, 33,37

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/06548

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/12/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4163778	07/08/79	NONE	

For more details about this annex: see Official Journal of the European patent Office, No. 12/82

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